Thermogenic ingredients : energy expenditure and intestinal absorption

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Energy expenditure and intestinal absorption

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Chapter 1

General introduction

Energy homeostasis is reached when energy intake derived from food and energy expenditure for body functions and physical activity, are in balance. This is needed to maintain body weight. However, changes in body weight occur when there is an imbalance between energy intake and energy expenditure. When energy intake exceeds energy expenditure, the excessive energy will be stored as fat, which will consequently result in body weight gain. Over the past years, the prevalence of obesity has increased in western society and developing countries (1-4), and in an environment that is in abundance of easy accessible and energy-dense food it is not easy to change the energy balance (5-7). Hence, investigating food ingredients that produce reductions in energy intake and absorption and promote energy expenditure has considerable importance for anti-obesity therapy. Capsaicin and catechin are such food ingredients, which affect energy intake and/or energy expenditure. Besides these effects on energy intake and expenditure, these food ingredients may also play a role in body weight regulation through interactions with the gut microbiota (8).

Energy intake

Food intake regulation and appetite control

The macronutrients carbohydrate, protein, fat and alcohol provide energy for the human body. As mentioned before, to maintain body weight it is of importance that energy intake meets the energy requirement, which is set by energy expenditure. For this reason energy intake, and thus food intake needs to be highly regulated. The daily energy requirements can be calculated based on basal metabolic rate (BMR) using the equation of Harris-Benedict (9), multiplied by the physical activity level (PAL) (10).

The brain regions that are involved in the control of food intake are hypothalamus, caudal brainstem, and parts of the cortex and limbic system (11). Two regulation systems have been described in controlling food intake: the homeostatic regulation system and the non-homeostatic regulation system. The homeostatic regulation system uses signals from the gastro-intestinal tract (e.g. the hormones leptin, ghrelin, glucagon-like peptide 1 (GLP1) and peptide YY (PYY)), metabolism and storage to regulate energy balance. In this system energy intake is related to hunger and satiety. Anorexigenic hormones leptin, insulin, GLP1, PYY and cholecystokinin inhibit the effect of hunger signals, while the orexigenic hormones ghrelin and neuropeptide Y increase hunger. Both anorexigenic and orexigenic hormones control food intake and energy balance by feedback from the gastro-intestinal tract to neurons in the hypothalamus. Energy intake can be measured by analyzing the actual *ad libitum* food intake, while

appetite, in terms of feelings of hunger, fullness, satiety and desire to eat can be measured with the use of anchored 100-mm visual analogue scales (VAS).
Non-homeostatic regulation is food intake driven by cognitive and environmental factors (including availability, palatability, social context, rewards). This deals mainly with cognitive, motivational and emotional aspects of ingestive behavior (12). In non-homeostatic eating, the degree to which overeating takes place has been attributed to the rewarding value of food (13,14), which can be divided into two processes: "liking" which is the hedonic preference for a food item and "wanting" which is the motivation to eat a food item (15). It has been suggested that the hormone ghrelin can act in a homeostatic as well as a non-homeostatic way, as it also interacts with mesolimbic areas involved in non-homeostatic (reward) feeding (16).

Effects of thermogenic ingredients on energy intake

Capsaicin, the major pungent component of chili pepper (*Capsicum, Solanaceae*) is a thermogenic ingredient which may affect energy intake and appetite profile. Several studies have shown that capsaicin decreases *ad libitum* food intake (17-19), enhances feelings of satiety and fullness in energy balance (18,20), and decreases feelings of desire to eat (21) and hunger (18) in energy balance as well as in positive energy balance (22). Furthermore, it may increase concentrations of GLP1 and decrease concentrations of ghrelin (23). When placed in the mouth, capsaicin binds to receptors on neurons found on the tongue that are sensitive to heat and pain; after binding, neurotransmitters are released, and a sensation of warmth or burning is felt (24). To quantify the pungency of peppers, the Scoville test is used (25). The number of Scoville heat units (SHU) indicates the amount of capsaicinoids present in the pepper. The capsaicin level in peppers can vary from plant to plant. Capsaicin can be ingested orally or in capsule form, with oral exposure being more effective to achieve the maximum effect, as energy intake is lower when capsaicin is ingested orally compared to administration in capsule form (18). Since protein is the most satiating macronutrient and has the largest diet-induced thermogenesis, the effect of capsaicin on energy intake and appetite profile may be enlarged when capsaicin is combined with a high protein diet. In negative energy balance, a relatively high protein intake has been shown to sustain satiety despite the negative energy balance (26). This protein-induced satiety may be due to elevated plasma amino acid concentrations, hunger suppression, and possibly increased anorexic hormone concentrations.

Energy expenditure and substrate oxidation

Energy expenditure

Energy expenditure consists of the four components diet-induced thermogenesis (DIT), sleeping metabolic rate (SMR), activity associated energy expenditure (AEE), and the energy cost of arousal (**Figure 1.1**). Sleeping metabolic rate and the energy cost of arousal together are defined as basal metabolic rate (BMR) or resting energy expenditure (REE) (27).

SMR is the energy expenditure measured during sleep and is measured as lowest mean energy expenditure measured over 3 consecutive hours during sleep. DIT, or the thermic effect of food, is the energy cost of digestion, absorption and conversion of ingested food. AEE is the energy cost of physical activity, this is the energy expenditure associated with muscular contractions to perform body movements. Finally, BMR accounts for the largest proportion of TEE and is the energy expenditure for normal body functions and homeostasis. Moreover, BMR is the energy expenditure of a person at rest, while being awake, in fasted state and in a thermoneutral environment (28).

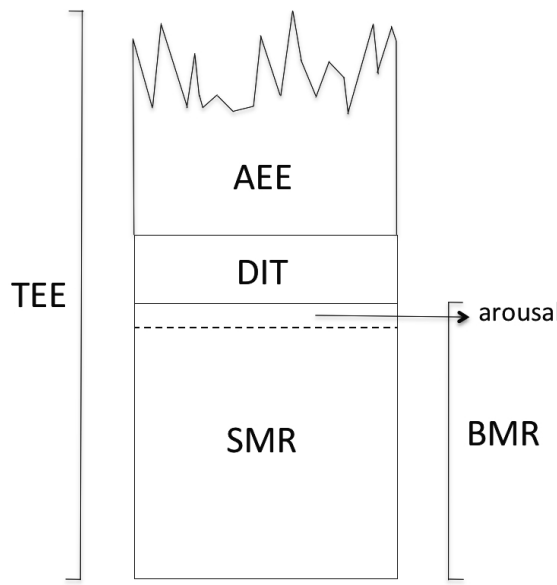

Figure 1.1 Total energy expenditure (TEE) with components: basal metabolic rate (BMR), which consists of sleeping metabolic rate (SMR) and energy costs of arousal, diet-induced energy expenditure (DIT), and activity associated energy expenditure (AEE).

Energy expenditure can be measured by oxygen consumption and/or carbon dioxide production. This is called indirect calorimetry, and can be carried out with a ventilated hood system or in a respiratory chamber. A respiration chamber is an airtight room, used for measuring whole body energy and substrate metabolism. This room is ventilated with fresh air, to measure energy expenditure from ventilation rate and oxygen and carbon dioxide concentrations from air flowing in and out (29). Ventilated hood is a plastic canopy around the head, also used to measure energy expenditure from ventilation rate and oxygen and carbon dioxide concentrations from air flowing in and out. Ventilated hood system is used for measuring REE and DIT and is calculated using formula of Weir (30).

Basic Weir equation:
Kilojoules (kJ) = ((3.9 VO2 used) + ((1.1 VCO2 produced))

Substrate oxidation

As highlighted in the previous paragraph, the macronutrients carbohydrate, protein, fat and alcohol form the substrates to provide energy for the body. The energy contained by these substrates is converted into adenosine triphosphate (ATP) and heat. ATP is used for all processes in the body requiring energy. Carbon dioxide produced per unit of oxygen consumed varies with the type of substrate catabolized. For this reason substrate oxidation can be calculated from O^2 consumption and CO^2 production. The respiratory quotient (RQ) describes the ratio of the metabolic gas exchange CO^2 produced / O^2 consumed, and indicates which substrate is being oxidized, as the RQ for carbohydrate equals 1.00 and RQ for fat equals 0.696 (31).

Effects of thermogenic ingredients on energy expenditure

Thermogenic food ingredients such as capsaicin, catechin and caffeine may stimulate energy expenditure, while they add only negligible amounts of energy to food intake. Although, there may be more food ingredients that cause an increase in energy expenditure, those ingredients seem to be less promising than the aforementioned ingredients. Capsaicin has been shown to stimulate thermogenesis by increasing energy expenditure (32-36). Furthermore, a decrease in RQ (34) and an increase in fat oxidation was found (32,33). This thermogenic effect of capsaicin may be caused by stimulation of the transient receptor potential vanilloid receptor (TRPV1). Besides allowing humans to detect an oral burn after consumption of red pepper, this capsaicin receptor may also have a role in thermoregulation (24,37).

Green tea (GT) may as well have an effect on energy balance, as it is rich in polyphenols, mainly catechins, which may play a role in body weight regulation. Green

tea is made from the plant Camellia sinensis L. and contains 10-20% catechins (38). Catechins are reported to increase fat oxidation (39,40), and energy expenditure (39-43) by inhibiting catechol-O-methyltransferase (COMT) (44), which is an enzyme that is responsible for degrading catecholamines. There seems to be a difference in sensitivity to catechins between ethnic groups, as in some studies with Caucasian subjects, no effects were seen after ingestion of green tea, while studies in Asians seem to report more favorable results (45,46). This difference in sensitivity to green tea catechins may be caused by the existence of a common *Val108/158Met* polymorphism of the COMT gene, since Asians have a higher frequency of the thermostable, high activity enzyme, $COMT^H$ allele, while Caucasians have nearly equal frequencies of the $COMT^H$ allele and $COMT^L$ allele (47). Finally, green tea also contains caffeine, which may stimulate thermogenesis and fat oxidation (48,49) via inhibition of the enzyme phosphodiesterase (50).

Body composition

Body composition refers to quantifiable components of the body including fat mass (FM), fat free mass (FFM), total body water (TBW), protein and bone. The general model for body composition is the two-compartment model. This model divides the body in two parts with a different density: FM with a density of 0.9 g/ml and FFM (water, proteins and minerals) with a density of 1.1 g/ml. Body composition has a substantial impact on energy expenditure, as FFM is the major single determinant of REE (51). In negative energy balance, it is to be preferred to lose primarily fat mass and hardly any fat free mass.

Effects of thermogenic ingredients on body composition

Long-term effects of green tea catechins have shown a favorable effect on body composition, as they have been reported to induce body weight (52-56) and body fat loss (52,54,55). Furthermore, several animal and human studies found an inhibiting effect of green tea catechins on lipase activity, which could cause a decrease in fat absorption (57-60). With decreased fat absorption, metabolizable energy is decreased, digestion efficiency is reduced, and fat excretion into feces is increased, which consequently may result in a decrease in body weight. Results on effects of capsaicin on body weight loss are limited. A study on the effects of capsaicin on weight maintenance after weight loss did not show a significant difference on weight regain after weight loss between capsaicin and control (32).

Gut microbiota

The human body consists of more microbial cells than human cells (61). About 1.5 kg of bacteria normally colonize the human body, in which they have immunologic, metabolic and barrier effects. These microorganisms are present in various parts of the human body, including the skin, vagina and respiratory and gastrointestinal tracts, and in each part the composition and function is different. The human gut, and especially the colon, is the most densely colonized body site containing trillions of bacteria, archaea and eukaryotic microorganisms (62). The human colonic microbiota constitutes a complex ecosystem, in which the majority of bacteria belong to the four phyla; *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (62). The phyla *Bacteriodetes* and *Firmicutes* are dominant in the large intestines (63).

Gut microbiota and body weight

There is a possible link between the human gut microbiota and body composition, as several studies have shown that lean and obese people have a different gut microbiota (64-66). When comparing the bacterial diversity of obese and normal weight people, a lower bacterial diversity in obese people has been observed (67,68). Moreover, an increase in body weight of germ free mice occurred when fecal microbiota from obese people was transplanted into germ-free mice, while body weight did not increase in mice receiving microbiota from lean people (69). This suggests that the gut microbiota of obese people is more efficient in extracting energy from the diet and to store this energy as fat, resulting in an increase in body weight (70). Furthermore, the gut microbiota may also affect body weight regulation via metabolic signalling of short chain fatty acids through G-protein coupled receptors, to regulate appetite hormones and modulate inflammation (71).

Effects of thermogenic ingredients on gut microbiota

Although the composition of the human gut microbiota is rather stable in adults, changes in the diet may affect the bacterial composition (72,73), such as the weight lowering effect of polyphenols, which are thought to be related to changes in the composition of the gut microbiota (8). A major part of polyphenols may interact with the gut microbiota when they reach the intestines (74). The microbiota in the intestines metabolizes polyphenols, and the end products of this metabolism may consequently change the composition of the gut microbiota. Furthermore, the cleavage of glycosidic linkages in polyphenols generates glycans that are important as a nutrient foundation for the gut microbiota (75) especially for the *Bacteroidetes*, as they are supposed to have a higher glycan degrading capacity than *Firmicutes*. Therefore polyphenols may alter the balance between these two phyla in favor of

Bacteroidetes (8). The effects of green tea catechins on human gut microbiota are still poorly understood; only a few studies suggest that catechins might inhibit pathogenic bacteria, and stimulate beneficial bacteria (76-81).

Outline thesis

Thermogenic food ingredients may have beneficial anti-obesity effects as they may induce body weight loss via an increase in energy expenditure, decrease in appetite, decease in fat absorption or via interactions with the gut microbiota.
Normally, introduction of a negative energy balance by reducing energy intake also decreases energy expenditure (82) and increases appetite (83). This often results in weight cycling (yo-yo effect) which is the principle of repeated weight loss and regain (84). At first the question is addressed whether acute effects (24 h) of the thermogenic ingredient capsaicin, during negative energy balance would counteract this decrease in energy expenditure, and increase in appetite and energy intake during negative energy balance (**chapter 2 and 3)**. As protein is the most satiating macronutrient, these effects of capsaicin in negative energy balance may be enlarged when capsaicin is combined with a diet high in protein. Consequently, **chapter 4** describes the acute effects of capsaicin in combination with a diet in which carbohydrate is partly replaced by protein on appetite, energy intake and energy expenditure, in negative energy balance.

Furthermore, there seems to be a difference in response to thermogenic ingredients between ethnic groups. Studies on effects of green tea on energy expenditure in Asians seem to report more favorable results than studies in Caucasians. These differences in sensitivity to green tea catechins may be caused by the existence of a common *Val108/158Met* polymorphism of the COMT gene, as Asians have a higher frequency of the high activity $COMT^{H}$ allele, while Caucasians have nearly equal frequencies of the $COMT^{H}$ allele and the low activity $COMT^{L}$ allele. **Chapter 5** aimed to determine the role of genetic predisposition in the effects of green tea catechins on energy expenditure and fat oxidation.

In addition to the inhibition of this enzyme COMT, another important mechanism behind the anti-obesity effects of green tea catechins may be a decrease in dietary fat absorption, as an increase in fecal fat excretion over the short-term was observed (60). These long-term effects of green tea supplementation on fat absorption and energy expenditure were discussed in **chapter 6**. Since a major part of green tea catechins may interact with the gut microbiota when they reach the intestines, **chapter 7** presents the role of the gut bacterial composition in the effects of long-

term supplementation with green tea catechins on energy extraction. Finally, **chapter 8** presents a general discussion of the results and implications for future research.

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Chapter 2

Acute effects of capsaicin on energy expenditure and fat oxidation in negative energy balance

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Abstract

Introduction
Addition of capsaicin (CAPS) to the diet has been shown to increase energy expenditure, therefore capsaicin is an interesting target for anti-obesity therapy.

Objective
We investigated the 24h effects of CAPS on energy expenditure, substrate oxidation and blood pressure during 25% negative energy balance.

Methods
Subjects underwent four 36h sessions in a respiration chamber for measurements of energy expenditure, substrate oxidation and blood pressure. They received 100% or 75% of their daily energy requirements in the conditions '100%CAPS', '100%Control', '75%CAPS' and '75%Control'. CAPS was given at a dose of 2.56 mg (1.03 g of red chili pepper, 39,050 Scoville heat units (SHU)) with every meal.

Results
An induced negative energy balance of 25% was effectively a 20.5% negative energy balance due to adapting mechanisms. Diet-induced thermogenesis (DIT) and resting energy expenditure (REE) at 75%CAPS did not differ from DIT and REE at 100%Control, while at 75%Control these tended to be or were lower than at 100%Control (p=0.05 and p=0.02 respectively). Sleeping metabolic rate (SMR) at 75%CAPS did not differ from SMR at 100%CAPS, while SMR at 75%Control was lower than at 100%CAPS (p=0.04). Fat oxidation at 75%CAPS was higher than at 100%Control (p=0.03), while with 75%Control it did not differ from 100%Control. Respiratory quotient (RQ) was more decreased at 75%CAPS (p=0.04) than at 75%Control (p=0.05) when compared with 100%Control. Blood pressure did not differ between the four conditions.

Conclusions
In an effectively 20.5% negative energy balance, consumption of 2.56 mg capsaicin per meal supports negative energy balance by counteracting the unfavorable negative energy balance effect of decrease in components of energy expenditure. Moreover, consumption of 2.56 mg capsaicin per meal promotes fat oxidation in negative energy balance and does not increase blood pressure significantly.

Introduction

Obesity is a result of an energy imbalance that develops when energy intake exceeds energy expenditure. Overweight and obesity are the fifth leading risk for global deaths, at least 2.8 million adults die each year as a result of being overweight or obese (1). Capsaicin, the major pungent principle of red chili pepper, is a thermogenic ingredient which stimulates energy expenditure and contains negligible amounts of energy itself. Therefore, capsaicin may be an interesting target for anti-obesity therapy. Several studies have shown that capsaicin stimulates thermogenesis by increasing the energy expenditure (2-6). Furthermore, a decrease in RQ (4) and a beneficial effect of capsaicin on fat oxidation was found (2,3).
Capsaicin is one of the five naturally occurring capsaicinoids: capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin. The number of Scoville heat units (SHU) indicates the amount of capsaicinoids present in the pepper. If a pepper contains 50,000 SHU, this means that its alcoholic extract needs to be diluted 1:50,000 to be pungent on the human tongue (7). Red chili pepper can be ingested orally or in capsule form, whereby oral exposure is relatively more effective with respect to thermogenesis (8). One explanation for effects of capsaicin may be that it produces pain and stimulates thermogenesis caused by stimulating the Transient Receptor Potential Vanilloid receptor 1 (TRPV1) (7,9). Another explanation may be that capsaicin causes an increase in catecholamine (epinephrine, norepinephrine and dopamine) secretion and sympathetic nervous system (SNS) activity and consequently, increases blood pressure (4,10-14). Human studies have shown that capsaicin increased the diet-induced thermogenesis (3,4,10). Both human and animal studies investigated the effect of capsaicin after administration of β-adrenergic blockers such as propranolol, and showed that the thermogenic effect of capsaicin is reduced after administration of beta-adrenergic blockers (12,15). This implies that the increased thermogenesis by capsaicin is probably based on β-adrenergic stimulation. Whether, in negative energy balance, a reduction in energy expenditure can be prevented by consuming capsaicin, remains to be shown.

Taken together, since capsaicin supplementation adds only negligible amounts of energy to food intake while it increases energy expenditure at least in energy balance, it is of importance to study whether these characteristics may be used as a concept for prevention of the yo-yo effect when entering negative energy balance. Normally, introduction of a negative energy balance by reducing energy intake causes reduction in energy expenditure. We hypothesize that capsaicin supplementation vs. control during negative energy balance counteracts the normal decrease in energy expenditure. Thereby, capsaicin may increase fat oxidation relative to control. The aim of the present study was to investigate the 24h effects of capsaicin in 25% negative energy balance on energy expenditure and substrate oxidation. We investigated

whether the 24h effects of capsaicin in 25% negative energy balance counteracted the effects of a negative energy balance on energy expenditure and enlarged fat oxidation compared to 100% energy intake without capsaicin.

Methods

Subjects

Nineteen healthy Caucasian subjects, aged between 18-50 years, with a body mass index (BMI, kg/m²) between 20-30 were recruited for this study. Subjects were recruited by advertisements in local newspapers and on notice boards at Maastricht University. All subjects underwent a medical screening; during this screening, subjects underwent anthropometric measurements, and completed questionnaires related to health, smoking behaviour, use of medication, alcohol consumption, physical activity and eating behaviour.

The inclusion criteria, besides an age between 18-50 years and a BMI between 20-30 kg/m², were a good health, non-smoking, not using a more than moderate amount of alcohol (<10 consumptions per week) or caffeine-containing beverages (<2 cups per day). Subjects had to be weight stable (weight change <3 kg during the last 6 months), not using medication except for oral contraceptives in women and had to be dietary unrestraint. The Three Factor Eating Questionnaire (TFEQ) was used to determine eating behaviour (16). Only non-restrained eaters (<10 scores on factor 1), these are persons who are not consciously occupied with food or who are caloric restricted, were selected. Subjects had to be moderately active (<5 hours exercise per week) and used to consuming spicy foods on a regular basis (1-2 days per week, in a low dosage with one meal/day). Pregnant or lactating women were also excluded. Individuals with allergies for the food items used in the study were excluded from participation. Subject sample size was calculated where α was 0.05, β was 0.95 using energy expenditure changes from past papers (11) to calculate the effect size. The sample size was finalized as 14 subjects. The α-level was two-sided.
A written informed consent was obtained from all the participants. The Medical Ethics Committee of the Academic Hospital in Maastricht approved the study. The study was registered as follows: Nederlands Trial Register, registration number NTR2944.

Study protocol

The study had a single-blinded, randomized crossover design with four randomly sequenced experimental conditions. Subjects underwent four 36h sessions in a respiration chamber for measurements of energy expenditure and substrate

oxidation. Two days prior to each session, subjects were provided with a standardized diet to consume at home in order to be fed in energy balance (energy % protein/fat/carbohydrate: 15/30/55), and to receive the same macronutrient proportions as during the respiration chamber experiment. The subjects were instructed to maintain their habitual activity level on the two days before each visit. Subjects were asked to abstain from alcohol consumption on the two days before each visit. Furthermore, they were asked not to drink caffeine after 10:00 PM on the day before each visit. The four test sessions were conducted at least one week apart for male subjects and four weeks apart for female subjects to prevent possible treatment-induced effects and to take possible effects of menstrual cycle phase on energy intake and energy expenditure in women into account.

Energy expenditure

Oxygen consumption and carbon dioxide production were measured in a respiration chamber (17). The respiration chamber is a 14 m^3 room, which is furnished with a bed, chair, computer, television, radio, telephone, intercom, sink and toilet. The room was ventilated with fresh air at a rate of 70-80 l/min. The ventilation rate was measured with a dry gas meter (type 4; Schlumberger, Dordrecht, Netherlands), and concentrations of oxygen and carbon dioxide were measured with the use of an infrared carbon dioxide analyzer (Uras 3G; Hartmann and Braun, Frankfurt, Germany and 2 paramagnetic oxygen analyzers: Magnos 6G; Hartmann and Braun, and type OA184A; Servomex, Crowborough, United Kingdom). During each 15-min period, 6 samples of outgoing air for each chamber, 1 sample of fresh air, zero gas, and calibration gas were measured. The gas samples to be measured were selected by a computer that also stored and processed the data. Total energy expenditure (=TEE) consists of SMR, DIT and activity-induced energy expenditure (AEE). 36h energy expenditure and 36h RQ were measured from 08:00h on the day subjects enter the respiration chamber to 20:00h on the next day. SMR was defined as the lowest mean energy expenditure measured over 3 consecutive hours between 00:00h and 07:00h. REE was calculated by plotting energy expenditure against radar output, that are both averaged over 30-min periods. The intercept of the regression line at the lowest radar output represents the energy expenditure in the inactive state (=REE), which consists of SMR and DIT. DIT was determined by subtracting SMR from REE. AEE was determined by subtracting SMR and DIT from TEE.

Substrate oxidation

Carbohydrate, fat, and protein oxidation were calculated from the measurements of oxygen consumption, carbon dioxide production, and urinary nitrogen excretion by using the formula of Carpenter in Brouwer *et al*. (18). Urine samples were collected

from the second void on the day subjects entered the respiration chamber to 20:00h on the next day. Samples, 3 per 36 hrs, in order to determine substrate oxidation were collected in containers with 10 ml HCl to prevent nitrogen loss through evaporation. Volume and nitrogen concentration were measured, the latter with a nitrogen analyzer (CHN-O-Rapid; Heraeus, Hanau, Germany). Urinary nitrogen was collected to calculate the RQ and protein balance correctly.

Blood pressure

Blood pressure (BP) was measured 15 minutes before each meal; these measurements were taken in sitting position and were made before the meal to avoid variability due to recent (within 2-3 h) food intake (19). Subjects were instructed to perform triplicate measurements 15 minutes before breakfast, lunch and dinner and were asked to record the mean of these three measurements.

Body composition

Body weight was measured using a digital balance and height was measured using a wall-mounted stadiometer. BMI was calculated as body weight (kg) divided by height (m) squared. The deuterium dilution method according to the Maastricht protocol (20) was used to measure total body water (TBW). The subjects were asked to collect a urine sample in the evening just before drinking a deuterium-enriched water solution. After ingestion of this solution, the subject went to bed and no additional consumption was allowed for this period of time. Ten hours after drinking the water solution, another urine sample was collected. The dilution of the deuterium isotope is a measure of the TBW of the subject. Fat mass (FM) was calculated as body weight minus TBW divided by the hydration factor 0.73. Additionally, FM was determined by Bodpod (21) measurements. Fat mass index (FMI) was calculated by FM (kg) divided by height (m) squared. BMI, FM (%) and FMI were used to define body composition. Waist and hip circumference were determined in standing position by a tape measure. Waist circumference was measured at the smallest circumference between rib cage and iliac crest, and hip circumference at the level of the spina iliaca anterior superior. Accordingly, waist-to-hip ratio (WHR) was calculated by dividing waist by hip circumference. Both waist circumference and waist-to-hip ratio were used to define different patterns of body fat distribution.

Energy intake and food choice

Subjects were fed in energy balance during two days before the test sessions. Subject specific daily energy requirements were calculated based on basal metabolic rate (BMR), which was individually calculated with the equation of Harris-Benedict (22), and multiplied by a physical activity level (PAL) of 1.7. This PAL value of 1.7 represents

the average PAL of modern humans, which ranges from 1.5 to 2.0 (23). In our population in the south of the Netherlands the PAL value of 1.7 is the mean (range 1.6-1.8) of the subjects, with the subject characteristics assessed in the present study. The energy intake level was estimated as such that subjects were not in a positive or a negative energy balance before they entered the respiratory chamber. In the respiration chamber energy requirements were calculated based on a PAL of 1.35. Subject received 100% of their daily energy requirements in the conditions '100%Control' and '100%CAPS' (energy% protein/fat/carbohydrate: 15/30/55), and received 75% of their daily energy requirements in the condition '75%Control' and '75%CAPS' (energy% protein/fat/carbohydrate: 15/30/55). Energy intake was divided over the meals as 20% for breakfast, 40% for lunch, and 40% for dinner. Subjects had to completely finish all drinks and meals within 30 minutes. Negative energy balance in both 75% conditions was calculated by energy intake minus TEE divided by 100% of their daily energy requirements.

Dosage

Red chili pepper from the Capsicum frutescens L and Capsicum annuum L (McCormick; USA, capsaicin 2484 μ/g, nordihydrocapsaicin 278 μ/g and dihydrocapsaicin 1440 μ/g) was used as source for capsaicin. With respect to the daily dose for capsaicin, the daily value has not been established. The generally recommended daily dose is 1350 mg capsicum with 0.25% capsaicin (40,000 SHU). Capsaicin was given at a dose of 2.56 mg (1.03 g of red chili pepper, 39,050 SHU) with every meal. This dosage was based upon the maximal dosage given in previous studies and in our pre-test (2,8,24). Divided over three meals, a daily total dose of 7.68 mg CAPS was consumed by the subjects.
Preceding the study in the respiration chamber, several tests concerning pungency and spiciness were conducted. In a pre-test several food items were tested in combination with different dosages of red chili pepper. Before the dosage of red chili pepper in the food was determined, the pleasantness of taste, spiciness and pungency of different food products with red chili pepper were assessed to determine whether the amount of red chili pepper was tolerable. Based upon this pre-study the products we chose to offer during the experiment were; breakfast drink original with red chili pepper concentration of 2.0 g/l, pâté with 1.0 g red chili pepper/30 g, tomato juice with 2.0 g red chili pepper/l and pizza containing 2.0 g red chili pepper. The given dose of each component did not exceed the maximum tolerable dose among our subjects.

Data analysis

The Statistical Package for the Social Sciences (SPSS) 17.0 was used to perform univariate within-subject analyses. Repeated-measures ANOVA was used to determine possible differences in energy expenditure and its components, substrate

oxidation, energy balances and macronutrient balances within-subjects, between the four conditions. Step-down tests were used for pair wise comparisons, post hoc, including Bonferroni corrections. Shapiro-Wilk test was used to determine normality of the variables, these appeared to be normally distributed. All statistical tests are two-sided and differences are considered statistical significant if $p<0.05$. Values are expressed as means and standard deviations or standard errors.

Results

Subject characteristics

Nineteen healthy subjects (nine males, ten females) started the experiments; four subjects dropped-out due to agenda problems. Subjects were used to consuming spicy foods on a regular basis, in general they consumed red chili pepper once per week (0.25–0.5 grams of dried red pepper or 1–2 grams of fresh red pepper). Fifteen subjects (seven female and eight male) completed the four conditions; 100% CAPS, 100%Control, 75%CAPS and 75%Control; the subjects had a mean age of 29.7±10.8 y and a mean BMI of 23.3±2.9 kg/m^2 (**Table 2.1**).

Energy expenditure

In two conditions subjects received 100% of the daily energy requirements and in the other two conditions they received 75% of the daily energy requirements. Energy balance during 36 h in the 100%CAPS and the 100%Control conditions did not significantly differ from 0 (**Table 2.2**). During the 75%CAPS and 75%Control conditions negative energy balance was 20.5±1.4% respectively 19.2±1.3%.

Total energy expenditure (TEE) in 100%CAPS and 100%Control did not differ significantly. As expected, total energy expenditure (TEE) was higher in the 100% conditions than in the 75% conditions (**Table 2.2**); overall an effect on TEE was observed ($p=0.018$). With respect to the components of energy expenditure (EE), the following appeared. DIT in the 75%CAPS condition did not differ from DIT in the 100%Control condition, while DIT tended to be lower in 75%Control condition compared with 100%Control condition ($p=0.05$). Similarly, REE in the 75%CAPS condition did not differ from REE in the 100%Control condition, while REE was significantly lower in 75%Control condition compared with 100%Control condition ($p=0.02$).

Taken together, 75%CAPS did not differ from 100%Control with respect to DIT and REE, while at 75%Control DIT tended to be lower and REE was lower than at 100%Control. Likewise, 75%CAPS did not differ from 100%CAPS regarding SMR, while SMR at 75%Control was lower than SMR at 100%CAPS (p=0.04).

Table 2.1 Subject characteristics (mean values and standard deviations)

	Male (n=8)	Female (n=7)	Total (n=15)
Age (year)	26.8 ± 8.4	33.0 ± 12.9	29.7 ± 10.8
Height (m)	1.82 ± 0.05	1.65 ± 0.05	1.74 ± 0.10
Body weight (kg)	79.8 ± 10.0	61.2 ± 10.3	71.2 ± 13.7
BMI (kg/m^2)	24.0 ± 2.6	22.4 ± 3.1	23.3 ± 2.9
FMI (kg/m^2)	4.4 ± 2.0	6.8 ± 2.0	5.5 ± 2.3
FFMI (kg/m^2)	19.6 ± 1.5	15.5 ± 1.3	18.6 ± 3.6
WHR	0.80 ± 0.04	0.69 ± 0.04	0.75 ± 0.07
FM (kg)	14.6 ± 6.6	18.8 ± 6.0	16.6 ± 6.5
FFM (kg)	65.3 ± 6.9	42.4 ± 4.8	54.6 ± 13.1
Body fat (%)	17.8 ± 7.1	30.1 ± 4.5	23.6 ± 8.6
TFEQ F1	2.5 ± 2.6	3.1 ± 2.9	2.8 ± 2.7
TFEQ F2	4.9 ± 1.2	5.4 ± 3.4	5.1 ± 2.4
TFEQ F3	4.1 ± 4.3	5.9 ± 3.4	4.9 ± 3.9

BMI: Body mass index; FMI: Fat mass index; FFMI: Fat free mass index; WHR: Waist-to-hip ratio; FM: Fat mass; FFM: Fat free mass; TFEQ: Three Factor Eating Questionnaire; F1, cognitive restraint; F2, disinhibition; F3, hunger. #The TFEQ measures three different factors of human eating behaviour.

Table 2.2 Total energy expenditure, components of energy expenditure, energy intake, substrate oxidation and mean RQ during the four conditions (n=15).

	100%CAPS	100%Control	75%CAPS	75%Control
EI (MJ/d)	9.09 ± 0.4	9.09 ± 0.4	6.81 ± 0.3**##	6.81 ± 0.3**##
EB (MJ/d)	0.15 ± 0.2	0.22 ± 0.2	-1.81 ± 0.1**##	-1.71 ± 0.1**##
TEE (MJ/d)	8.82 ± 0.4	8.75 ± 0.4	8.52 ± 0.4*#	8.41 ± 0.4**#
REE (MJ/d)	7.70 ± 0.4	7.55 ± 0.3	7.49 ± 0.3	7.35 ± 0.3*#
SMR (MJ/d)	6.69 ± 0.3	6.47 ± 0.3	6.45 ± 0.3	6.39 ± 0.3*
DIT (MJ/d)	1.00 ± 0.1	1.09 ± 0.1	1.03 ± 0.1	0.95 ± 0.1#
AEE (MJ/d)	1.12 ± 0.1	1.20 ± 0.1	1.03 ± 0.1	1.06 ± 0.1
Fat oxidation (MJ/d)	1.63 ± 0.2	1.63 ± 0.2	2.38 ± 0.2*#	2.17 ± 0.2*
Carbohydrate oxidation (MJ/d)	5.89 ± 0.2	5.97 ± 0.2	5.03 ± 0.2**##	5.18 ± 0.2**##
RQ	0.92 ± 0.02	0.92 ± 0.02	0.89 ± 0.02**#	0.90 ± 0.01**#

EI: Energy intake; EB: Energy balance; TEE: Total energy expenditure; REE: Resting energy expenditure; SMR: Sleeping metabolic rate; DIT: Diet-induced thermogenesis; AEE: Activity-induced energy expenditure; RQ: Respiratory quotient.* p<0.05 compared to 100%CAPS, ** p<0.01 compared to 100%CAPS. # p<0.05 compared to 100%Control, ## p<0.01 compared to 100%Control.

Substrate oxidation

Addition of capsaicin to the meals significantly increased the 24h fat oxidation in negative energy balance. Fat oxidation in the 75%CAPS condition was significantly

higher than fat oxidation in the 100%Control condition (p=0.03), while at 75%Control fat oxidation did not differ significantly from fat oxidation at 100%Control. Carbohydrate oxidation in 75%CAPS and 75%Control were lower than in 100%Control (p<0.01 and p<0.01 respectively) and than 100%CAPS (p<0.001 and p<0.001 respectively).

An overall effect of protein balance was observed (p<0.001). Protein balance in the 100%Caps condition was significantly higher than in the 75%CAPS (p<0.01) and in the 75%Control condition (p<0.01); similar findings were present between 100%Control and both 75%conditions (75%CAPS p<0.01, 75%Control p<0.01). Fat balance was more negative in both 75%conditions vs. 100%conditions and seemed to be more negative in the 75%CAPS than in the 75%Control condition while carbohydrate balance was more negative in the 75%Control (p<0.01) than in the 75%CAPS (p<0.05) condition, vs. 100%Control. Separate macronutrient balances are shown in **Figure 2.1**.

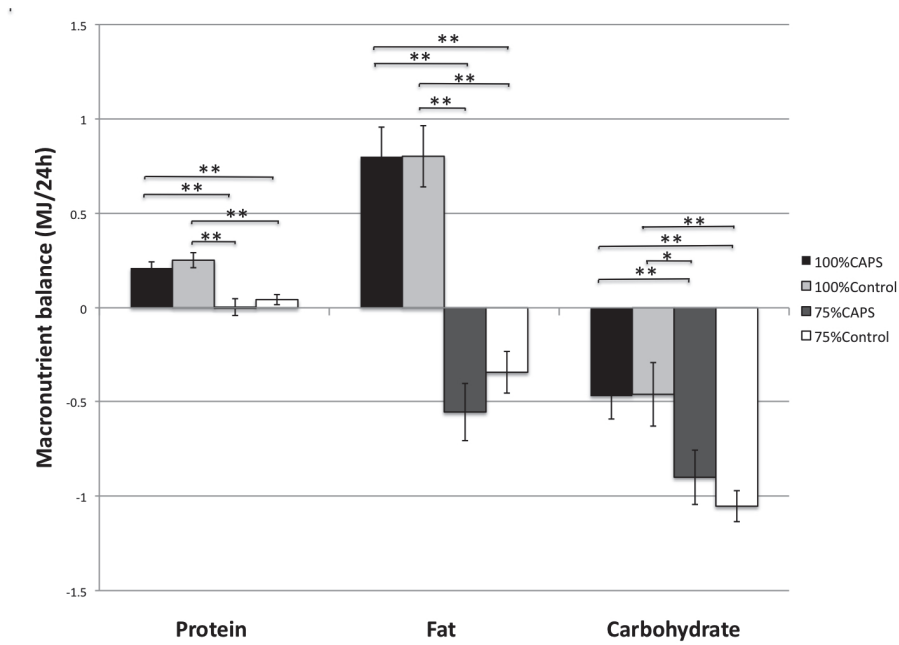

Figure 2.1 Macronutrient balances for 100%CAPS (black), 100%Control (light grey), 75%CAPS (dark grey) and 75%Control (white) conditions in fifteen subjects (seven female and eight male).

RQ

No significant differences in RQ were seen between the two conditions in energy balance (100%CAPS 0.92±0.02 and 100%Control 0.92±0.02) nor between the two conditions in negative energy balance (75%CAPS 0.89±0.02 and 75%Control 0.90±0.01). RQ was decreased in both 75%CAPS and 75%Control conditions when compared with 100%Control condition. However, RQ was significantly more decreased in the 75%CAPS condition (p=0.04) than in the 75%Control condition (p=0.05) when compared with 100%Control (**Table 2.2**).

Blood pressure

No significant differences were found when systolic and diastolic blood pressure readings were compared between the four conditions **(Table 2.3).**

Table 2.3 Systolic and diastolic blood pressure measurements for the conditions 100%CAPS, 100%Control, 75%CAPS and 75%Control as measured 15 minutes before the meals (n=15).

			Systolic (mmHg)		
Day	Moment in time	100%CAPS	100%Control	75%CAPS	75%Control
1	Before breakfast	116.1 ± 11.7	117.5 ± 7.2	119.9 ± 10.5	114.9 ± 10.5
	Before lunch	118.1 ± 11.3	118.5 ± 11.0	117.7 ± 14.5	113.5 ± 10.2
	Before dinner	114.4 ± 11.9	117.6 ± 10.6	120.8 ± 9.9	113.3 ± 12.2
2	Before breakfast	116.9 ± 12.9	118.5 ± 12.1	118.5 ± 10.5	116.3 ± 12.3
	Before lunch	116.1 ± 11.0	117.5 ± 9.3	119.9 ± 10.4	114.9 ± 13.8
	Before dinner	118.1 ± 10.8	118.5 ± 12.2	117.7 ± 11.4	113.5 ± 11.7
			Diastolic (mmHg)		
Day	Moment in time	100%CAPS	100%Control	75%CAPS	75%Control
1	Before breakfast	72.0 ± 7.9	70.6 ± 6.5	72.9 ± 7.9	69.5 ± 7.8
	Before lunch	71.4 ± 8.5	71.8 ± 8.7	70.5 ± 10.1	70.2 ± 7.5
	Before dinner	71.9 ± 9.9	70.9 ± 9.0	73.4 ± 8.7	69.3 ± 8.8
2	Before breakfast	72.7 ± 7.7	70.7 ± 8.9	69.6 ± 7.2	70.4 ± 10.8
	Before lunch	72.0 ± 8.3	70.6 ± 8.1	72.9 ± 9.3	69.5 ± 10.3
	Before dinner	71.4 ± 7.4	71.8 ± 7.5	70.5 ± 9.3	70.2 ± 10.1

Discussion

In the present study we tested the hypothesis that the 24h effects of capsaicin in 25% negative energy balance would counteract the effects of a negative energy balance on energy expenditure and enlarge fat oxidation compared to 100% energy intake without capsaicin. Therefore we investigated the effects of capsaicin on energy expenditure, substrate oxidation, macronutrient balance and RQ in 100% energy

balance and in 75% negative energy balance. Capsaicin was given at a dose of 2.56 mg capsaicin (1.03 g of red chili pepper, 39,050 SHU) with every meal. The induced negative energy balance of 25%, which was obtained by feeding 25% less energy than in energy balance, resulted in a real negative energy balance of 20.5% with CAPS, and of 19.2% with Control.

The strong negative energy balance with capsaicin was due to two main findings. First, DIT and REE in the 75%CAPS condition did not lead to a significant decrease compared with 100%Control, while DIT and REE tended to be or were significantly lower in the 75%Control condition compared with 100%Control. Thus, the effects of capsaicin vs. control in negative energy balance counteracted the effects of the negative energy balance on DIT and REE compared to 100% energy intake without capsaicin. Second, there was a significant increase in fat oxidation when capsaicin was added to the meals in negative energy balance, while there was no significant increase in fat oxidation in the 75%Control condition compared with the 100%Control condition. Since blood pressure did not differ between the four conditions, capsaicin contributed to counteracting effects of negative energy balance without a significant increase in blood pressure. In line with previous studies, that assessed administration of capsaicin in energy balance (3-6), we expected an increased TEE with 100%CAPS vs. 100%Control. However, this did not reach statistical significance, neither did the components of TEE, namely SMR and DIT. Before, we did observe a larger TEE with 100%CAPS vs. 100%Control (25); although in the present study differences were observed, lack of statistical significance may be due to the number of subjects included. A long-term study by Lejeune *et al.* on the effect of 135 mg capsaicin/d on body-weight regain after weight loss found no limiting effect on weight regain after weight loss, yet an increase in thermogenesis and fat oxidation (2). In this study and another study a similar finding on fat oxidation has been reported, these studies found that capsaicin increased fat oxidation over the long term (3 months) and over the short term (after one breakfast) (2,3). The effects of capsaicin on protein oxidation, fat oxidation and carbohydrate oxidation contribute to beneficial effects on body composition and herewith promote an increase in fat free mass and a reduction in FM (26). Although these beneficial effects on fat oxidation will not guarantee body weight loss or body-weight maintenance, they may counteract a decrease in EE. Yoshioka *et al.* found that the increase in fat oxidation after capsaicin administration was mainly observed when the meals had a high fat content (energy % protein/ fat/carbohydrate: 15/45/40) (3). In our study the fat content of the meals was normal (energy % protein/fat/carbohydrate: 15/30/55), thus the effect of capsaicin on fat oxidation might have been higher if we would have increased the fat content of the meals. The effects of capsaicin on EE and substrate oxidation do not seem to be acute, but to build up over a few days, since another one-meal study with Caucasians on the acute effect of red chili pepper on satiety, energy expenditure and substrate oxidation found no effect of capsaicin (24). Given its strong pungency, the long-term use of

capsaicin may be limited. A possible solution may be capsinoids. Capsinoids including 'capsiate' are non-pungent capsaicinoid analogues. Capsinoids have similar beneficial effects on energy expenditure and substrate oxidation to those of capsaicin (27).

In the present study the subjects received 3.09 g red chili pepper per day (7.68 mg capsaicin); this dosage is relatively low compared to dosages used in studies with Asians (3,10,28). However, there is a difference in maximum tolerable dose of red chili pepper between Asians and Caucasians. This difference in tolerable dose is due to the difference in red chili pepper consumption. Red chili pepper is more common in the food pattern in Asian population. For example, the capsaicin consumption in India is 25-200 mg/day while the average daily consumption in Europe is estimated to be 1.5 mg (29). Next to studies investigating effects of capsaicin in Caucasians (2,10,24,30), several studies that investigated the effects of capsaicin on appetite and energy intake have been conducted in Asian populations (3,10,28).

In summary, the present study shows that the effects of capsaicin vs. control in 25% negative energy balance did prevent the effects of the negative energy balance on DIT and REE compared to 100% energy intake without capsaicin. Moreover, it increased fat oxidation in negative energy balance. The presumed negative energy balance of 25% led to a negative energy balance of 20.5±1.4% when capsaicin was added to the meals and 19.2±1.3% without addition of capsaicin. Since DIT and REE at 75%CAPS were similar as DIT and REE at 100%Control, we conclude that in an effectively 20.5% negative energy balance consumption of 2.56 mg capsaicin per meal supports negative energy balance by counteracting the unfavorable negative energy balance effect of a decrease in components of energy expenditure. Moreover, consumption of 2.56 mg capsaicin per meal promotes fat oxidation in negative energy balance, and does not increase blood pressure significantly.

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Chapter 3

Capsaicin increases sensation of fullness in energy balance, and decreases desire to eat after dinner in negative energy balance

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Abstract

Introduction
Addition of capsaicin (CAPS) to the diet has been shown to increase satiety; therefore CAPS is of interest for anti-obesity therapy.

Objective
We investigated the effects of CAPS on appetite profile and *ad libitum* energy intake in relation to energy balance.

Methods
Fifteen subjects (seven women and eight men, age: 29.7±10.8yrs, BMI: 23.3±2.9 kg/m^2) underwent four conditions in a randomized crossover design in 36 hour sessions in a respiration chamber; they received 100% of their daily energy requirements in the conditions '100%Control' and '100%CAPS', and 75% of their daily energy requirements in the conditions '75%Control' and '75%CAPS', followed by an *ad libitum* dinner. In the 100%CAPS and 75%CAPS conditions CAPS was given at a dose of 2.56 mg (1.03 g of red chili pepper, 39,050 Scoville heat units) with every meal.

Results
Satiety ($p<0.05$) and fullness ($p=0.01$) were measured every waking hour and before and after every meal using visual analogue scales, and were higher in the 100%CAPS vs. 100%Control condition. After dinner desire to eat, satiety and fullness did not differ between 75%CAPS and 100%Control, while desire to eat was higher ($p<0.05$) and satiety ($p=0.06$) and fullness ($p=0.06$) tended to be lower in the 75%Control vs.100%Control condition. Furthermore, *ad libitum* intake ($p=0.07$) and overconsumption ($p=0.06$) tended to decrease in 100%CAPS vs. 100%Control.

Conclusions
In energy balance, addition of capsaicin to the diet increases satiety and fullness, and tends to prevent overeating when food intake is *ad libitum*. After dinner, capsaicin prevents the effects of the negative energy balance on desire to eat.

Introduction

Obesity is the result of an energy imbalance that develops when energy intake exceeds energy expenditure. Obesity raises the risk of developing Type 2 Diabetes, cardiovascular diseases and several cancers (1). Capsaicin (CAPS) is the major pungent principle of red chili pepper, which limits energy intake while it contains only negligible amounts of energy itself (2-4). Therefore, CAPS might be an interesting target for anti-obesity therapy. To quantify the pungency of hot peppers, the Scoville test is used; the number of Scoville heat units (SHU) indicates the amount of CAPS present in the pepper. The CAPS level in peppers can vary from plant to plant. If a pepper contains 50,000 SHU, this means that its alcoholic extract needs to be diluted 1:50,000 to be pungent on the human tongue (5). Red chili pepper can be ingested orally or in capsule form. In a previous study on the relative oral and gastrointestinal contribution to capsaicin-induced effects on food intake, the decrease in energy intake was larger when red chili pepper was ingested orally compared with administration in capsule form. So, oral exposure to red chili pepper is more effective to achieve the maximum effect (3).

Several human studies have shown that the addition of CAPS to a diet enhances anorexigenic sensations, including satiety and fullness in energy balance (3,6). Moreover, CAPS has been reported to suppress orexigenic sensations, it might decrease desire to eat (2) and hunger (3) in energy balance as well as in positive energy balance (23). In addition, several studies have shown that CAPS decreases *ad libitum* food intake (2-4). The exact mechanism of action is not fully understood; however, CAPS has been shown to increase concentrations of the anorexigenic hormone glucagon-like peptide 1 and to decrease concentrations of the orexigenic hormone ghrelin (7).

Furthermore, a study in rats using a diet containing CAPS has shown that CAPS causes an increase in catecholamine secretion from the adrenal medulla (8). This catecholamine secretion is caused by activation of the central nervous system (9). There is an association between sympathetic nervous system (SNS) activity and food intake behavior; food intake decreases when SNS activity increases (10). The increase in SNS activity after ingestion of CAPS suggests that the reduction in energy intake could be due to the anorexigenic effect of catecholamines (4).

Yoshioka *et al.* (4) found that the addition of red chili pepper to breakfast decreased the protein and fat intake at lunchtime; these beneficial effects of CAPS on food consumption were observed when the meals had a high fat content (energy % protein/fat/carbohydrate: 15/45/40). It still is necessary to assess whether there is a beneficial effect when CAPS is added to meals with a normal protein, fat and

carbohydrate content (energy % protein/fat/carbohydrate: 15/30/55). Furthermore, the study of Yoshioka *et al.* (4) was conducted in Asians with relatively high doses of red chili pepper (10 g of red chili pepper per meal). There is a difference in maximum tolerable dose of red chili pepper between Japanese and Caucasians. This difference in tolerable dose is due to the difference in habitual red chili pepper consumption. In many Asian countries, red chili pepper is more common in the food pattern. For example, the average CAPS consumption in India is 25-200 mg/day while the average daily consumption in Europe is estimated to be 1.5 mg (11). We investigated the effect of CAPS in Caucasians and with lower doses of red chili pepper (1.03 g of red pepper per meal). For weight-reduction strategies it is necessary to investigate whether these effects of CAPS are still present during a moderate energy deficit. Since CAPS supplementation adds only negligible amounts of energy to food intake while it may enhance anorexigenic sensations and suppress orexigenic sensations, at least in energy balance, it is of importance to study whether these characteristics may be used as a concept for prevention of the yo-yo effect when entering negative energy balance. Yo-yo effect (or weight cycling) is the principle of repeated weight loss and regain (12). Normally, introduction of a negative energy balance by reducing energy intake causes an increase in appetite (13). We assessed whether CAPS supplementation vs. control during negative energy balance would counteract the normal increase in appetite. Taken together, we investigated whether there is an effect of CAPS on appetite profile and *ad libitum* energy intake in Caucasians when 1.03 g of red pepper is added to meals with a normal fat and normal protein content in a 25% negative energy balance.

Methods

Subjects

Nineteen healthy Caucasian subjects, aged between 18-50 years and a body mass index (BMI) between 20-30 kg/m² were recruited for this study. Subjects were recruited by advertisements in local newspapers and on notice boards at Maastricht University. All subjects underwent a medical screening, including anthropometric measurements, and questionnaires related to health, smoking behavior, use of medication, use of dietary supplements, alcohol and caffeine consumption, physical activity, habitual red chili pepper intake, eating behavior, mood and anxiety.
The inclusion criteria, besides an age between 18-50 years and a BMI between 20-30 kg/m², were good health, non-smoking, not using dietary supplement and medication except for oral contraceptives in women, not using a more than moderate amount of alcohol (less than 10 alcoholic drinks (10 g alcohol per drink) per week) or caffeine-containing beverages (less than 2 cups per day). Subjects had to be weight

stable (weight change <3 kg during the last 6 months) and dietary unrestrained. The Three Factor Eating Questionnaire was used to determine eating behavior (14). Only non-restrained eaters (<10 scores on factor 1) who are not consciously occupied with food or who are caloric restricted, were selected (14). Subjects had to be lightly or moderately active (1-5 hours moderate exercise per week) and used to consuming spicy foods on a regular basis (1-2 days per week, in a low dosage with one meal/day). In general, they consumed red chili pepper once per week (mean intake: 0.25-0.5 grams of dried red pepper or 1-2 grams of fresh red pepper). Pregnant or lactating women were also excluded. Individuals with allergies for the food items used in the study were excluded from participation.
This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by The Medical Ethics Committee of the Academic Hospital in Maastricht. The study was conducted in de metabolic unit of Maastricht University, department of Human Biology and was registered as follows: ISRCTN, registration number NTR2944.

Subject characteristics

Body weight was measured using a digital balance and height by a wall-mounted stadiometer. BMI was calculated as body weight (kg) divided by height (m) squared. The deuterium dilution method according to the Maastricht protocol (15) was used to measure total body water (TBW). The subjects were asked to collect a urine sample in the evening just before drinking a deuterium-enriched water solution. After ingestion of this solution, the subject went to bed, and no additional consumption was allowed. Ten hours after drinking the water solution, another urine sample was collected. The dilution of the deuterium isotope is a measure of the TBW of the subject. Fat mass (FM) was calculated as body weight minus TBW divided by the hydration factor 0.73. Additionally, fat mass was determined by BodPod measurements (air displacement plethysmography) (16). Fat mass index (FMI) was calculated by FM (kg) divided by height (m) squared. BMI, FM (%) and FMI were used to define body composition. Waist and hip circumference were determined in standing position by a tape measure. Waist circumference was measured at the smallest circumference between rib cage and iliac crest, and hip circumference at the level of the spina iliaca anterior superior. Accordingly, waist-to-hip ratio was calculated by dividing waist by hip circumference. Waist-to-hip ratio was used to define different patterns of body fat distribution (**Table 3.1**)**.**

Study protocol

The study had a single-blinded, randomized crossover design with four randomly sequenced experimental conditions. Subjects underwent four 36h sessions in a respiration chamber (17). Two days prior to each session, subjects were provided with

a standardized diet to consume at home in order to be fed in energy balance (energy % protein/fat/carbohydrate: 15/30/55), and to receive the same macronutrient proportions as during the experiment. The energy intake level was estimated as such that subjects were not in a positive or a negative energy balance before they entered the respiration chamber. Subjects were instructed to maintain their habitual activity level and were asked to abstain from alcohol consumption on the two days before each visit. Furthermore, they were asked not to drink caffeine after 22:00 hrs on the day before each visit. The four test sessions were conducted at least one week apart for male subjects and four weeks apart for female subjects to prevent possible treatment-induced effects and to take eventual effects of menstrual cycle phase on energy intake in women into account. The results on energy expenditure and substrate oxidation are published elsewhere (17).

Table 3.1 Subject characteristics.

	Male (n=8)	Female (n=7)	Total (n=15)
Age (years)	26.8 ± 8.4	33.0 ± 12.9	29.7 ± 10.8
Height (m)	1.82 ± 0.05	1.65 ± 0.05	1.74 ± 0.10
Body weight (kg)	79.8 ± 10.0	61.2 ± 10.3	71.2 ± 13.7
BMI (kg/m^2)	24.0 ± 2.6	22.4 ± 3.1	23.3 ± 2.9
WHR	0.80 ± 0.04	0.69 ± 0.04	0.75 ± 0.07
FM (kg)	14.6 ± 6.6	18.8 ± 6.0	16.6 ± 6.5
FFM (kg)	65.3 ± 6.9	42.4 ± 4.8	54.6 ± 13.1
Body fat (%)	17.8 ± 7.1	30.1 ± 4.5	23.6 ± 8.6
TFEQ F1	2.5 ± 2.6	3.1 ± 2.9	2.8 ± 2.7
TFEQ F2	4.9 ± 1.2	5.4 ± 3.4	5.1 ± 2.4
TFEQ F3	4.1 ± 4.3	5.9 ± 3.4	4.9 ± 3.9

BMI: Body mass index; WHR: Waist-to-hip ratio; FM: Fat mass; FFM: Fat free mass; TFEQ: Three Factor Eating Questionnaire; F1, cognitive restraint; F2, disinhibition; F3, hunger. #The TFEQ measures three different factors of human eating behaviour. Subjects were Caucasian, and from the surrounding area of Maastricht, the Netherlands. Values are means with their standard errors (SE).

Energy intake and food choice

Subjects were fed in energy balance during two days before the test sessions. Subject specific daily energy requirement was calculated based on basal metabolic rate, which was individually calculated with the equation of Harris-Benedict (18), and multiplied by a physical activity level (PAL) of 1.7. This PAL value of 1.7 represents the average PAL of modern humans, which ranges from 1.5 to 2.0 (19). In our population in the south of the Netherlands the PAL value of 1.7 is the mean (range 1.6-1.8) of the subjects, with the subject characteristics assessed in the present study. In the respiration chamber energy requirements were calculated based on a PAL of 1.35. The respiration chamber is a 14m³ room, in this chamber subjects are only able to perform activities such as sitting, reading, watching television, and using the computer. By

experience, the mean PAL of the subjects in the respiration chamber is 1.35. Subjects received 100% of their daily energy requirements in the conditions '100%Control' and '100%CAPS', and received 75% of their daily energy requirements in the condition '75%Control' and '75%CAPS'. Negative energy balance in both 75% conditions was calculated by energy intake minus total energy expenditure (TEE) divided by 100% of their daily energy requirements (17). Energy intake was divided over the meals as 20% for breakfast (08:30hrs), 40% for lunch (13:30 hrs), and 40% for dinner (18:30hrs). Subjects had to completely finish all drinks and meals within 30 minutes. On day 1, subjects were asked to go to bed and to switch off the lights at 23:30 hrs, and on day 2 they were awakened at 07:30 hrs.

Dosage

Red chili pepper from the Capsicum frutescens L. and Capsicum annuum L. (McCormick; USA, capsaicin 2484 μ/g, nordihydrocapsaicin 278 μ/g and dihydrocapsaicin 1440 μ/g) was used as source for CAPS. With respect to the daily dose for CAPS, the daily value has not been established. However, the generally recommended daily dose, as stated on labels on bottles with capsules available from health shops, is about 1350-4000 mg capsicum with 0.25% CAPS (40,000 SHU). In the CAPS conditions, CAPS was given at a dose of 2.56 mg (1.03 g of red chili pepper, 39.050 SHU) with every meal. This dosage was based upon the maximal dosage given in previous studies (3,7,20). Divided over three meals, a daily total dose of 7.68 mg CAPS was consumed by the subjects. Preceding the study in the respiration chamber, several tests concerning pungency and spiciness were conducted using anchored 100-mm visual analogue scales (VAS) (21). In a pre-test several food items were tested in combination with different doses of red chili pepper. Before the dosage of red chili pepper in the food was determined, the pleasantness of taste, spiciness and pungency of different food products with red chili pepper were assessed to determine whether the amount of red chili pepper was tolerable. Based upon this pre-study the products we chose to offer during the experiment were; breakfast drink (Hero, breakfast drink original) with red chili pepper concentration of 2.0 g/l, pâté (Kips, cremepaté) with 1.0 g red chili pepper/30 g, tomato juice (Appelsientje, Zontomaat) with 2.0 g red chili pepper/l and pizza (AH, cheese-tomato) containing 2.0 g red chili pepper/300 g. The given dose of each component did not exceed the maximum tolerable dose among our subjects. The ratings concerning the spiciness and pungency of these products with red chili pepper varied between 50-80 mm on the VAS, these scores represent a very spicy solution, but tolerable (3). Subjects were allowed to drink water *ad libitum* during their stay in the respiration chamber. Subjects recorded water intake using a measuring jug.

Appetite profile

Appetite profile (hunger, fullness, satiety and desire to eat) was measured using VAS (21). During each 36h session these questionnaires were completed every waking hour, and before and after every meal. The appetite VAS data are given as area under the curve (AUC). AUC is the area above the baseline, calculated by the conventional trapezoid rule. Furthermore subjects completed questionnaires on pleasantness of taste, heat sensation, pungency and spiciness of the food after every meal. The scale was anchored from 'not at all' on the left to 'extremely' on the right.

Ad libitum meal

On the second day of the stay in the respiration chamber, pizza (AH, cheese-tomato, 0.9 MJ per 100g; energy % protein/carbohydrate/fat: 15/50/35) was provided for dinner *ad libitum* and was weighed before it was offered to the subjects. After 30 minutes the leftover of the pizza was weighed again; then the amount of pizza consumed was calculated.

Overconsumption and overcompensation

Overconsumption for the 100%CAPS and the 100%Control conditions and overcompensation for the 75%CAPS and 75%Control conditions were calculated as the difference between the *ad libitum* meal and 100% of the daily energy requirement expressed as a percentage of the daily energy requirement.

Data analysis

The Statistical Package for the Social Sciences 20.0 (SPSS Inc, Chicago, IL) was used to perform univariate analyses. Repeated-measures ANOVA was used to determine possible differences in *ad libitum* energy and water intake, appetite profile, overconsumption and overcompensation between the four conditions. ANOVA repeated measures with sex as covariate was used to determine possible differences between male and female subjects. Post hoc comparisons were made using Bonferroni. All statistical tests are two-sided and differences are considered statistical significant if $p<0.05$. Values are expressed as means and standard deviations or standard errors. Data were normally distributed. The power calculation was based on a study of Yoshioka *et al*. (22), in which they investigated the combined effects of red pepper and caffeine on *ad libitum* intake in eight Caucasian subjects. Subject sample size was calculated using *ad libitum* energy intake (kJ) after dinner where α was 0.05, β was 0.10, mean change of respectively 6140±1470 kJ for red chili pepper and 4510±1640 kJ for control, and a dropout rate of 20% at least 18 subjects needed to be included at the start of the study.

Results

Subject characteristics

Nineteen healthy subjects (nine males, ten female) started the experiments; four subjects dropped out due to agenda problems. Fifteen subjects (7 females and 8 males) completed the four conditions. They had a mean age of 29.7±10.8 and a mean BMI of 23.3±2.9 kg/m^2. Energy balance during 36h in the 100%CAPS and the 100%Control conditions were 0.15±0.2 MJ/d and 0.22±0.2 MJ/d respectively and did not significantly differ from 0. During the 75%CAPS and 75%Control conditions negative energy balance was 20.5±1.4% respectively 19.2±1.3% (17). No significant differences were shown between men and women concerning appetite profile, *ad libitum* intake, overconsumption and overcompensation.

Appetite profile

Baseline ratings for appetite profile were not different between the four conditions. The appetite profile (**Table 3.2**) shows that the general satiety ($p<0.05$) and fullness ($p=0.01$, **Figure 3.1**) over 36h, expressed as AUC were significantly higher in the 100%CAPS condition compared to the 100%Control condition.

Table 3.2 Area Under Curve of hunger, fullness, satiety and desire to eat (mmVAS x 36h) during the four conditions.

	100%CAPS	100%Control	75%CAPS	75%Control
Hunger	67674.5 ± 3192.5^{a}	73741.8 ± 3541.6^{a}	88216.5 ± 3889.0^{b}	87299.8 ± 3768.4^{b}
Fullness	109576.6 ± 3378.2^{a}	99522.3 ± 2983.3^{b}	84663.5 ± 2327.7^{c}	91145.7 ± 2820.9^{bc}
Satiety	118185.3 ± 3677.9^{a}	101157.8 ± 3051.4^{b}	89337.5 ± 2772.8^{bc}	92689.8 ± 3023.8^{bc}
Desire to eat	72360.9 ± 3270.9^{a}	83546.7 ± 3763.3^{ab}	96834.5 ± 3982.0^{c}	99366.8 ± 4187.2^{bc}

$^{a–c}$ Mean values within a row with unlike superscript letters were significantly different $p<0.05$ (repeated measures ANOVA), $n=15$.

After dinner on day 1 desire to eat scores after 75% CAPS (26.6±7.7 mm) did not differ from 100%Control (21.1±5.2 mm), while desire to eat after 75%Control (35.2±7.0 mm) was significantly higher than after 100%Control (21.1±5.2 mm, $p<0.05$). Similar observations were present with satiety and fullness. Satiety and fullness were not different for 75%CAPS vs. 100%Control (satiety: 75%CAPS 69.5±5.4 mm, 100%Control 77.8±3.7 mm; fullness: 75%CAPS 67.7±5.6 mm, 100%Control 75.0±3.5 mm), while there was a trend toward lower feelings of satiety and fullness in 75%Control than in 100%Control (satiety: 75%Control 64.7±5.6 mm, 100%Control 77.8±3.7 mm ($p=0.06$), fullness: 75%Control 58.9±6.9 mm, 100%Control 75.0±3.5 mm ($p=0.06$)).

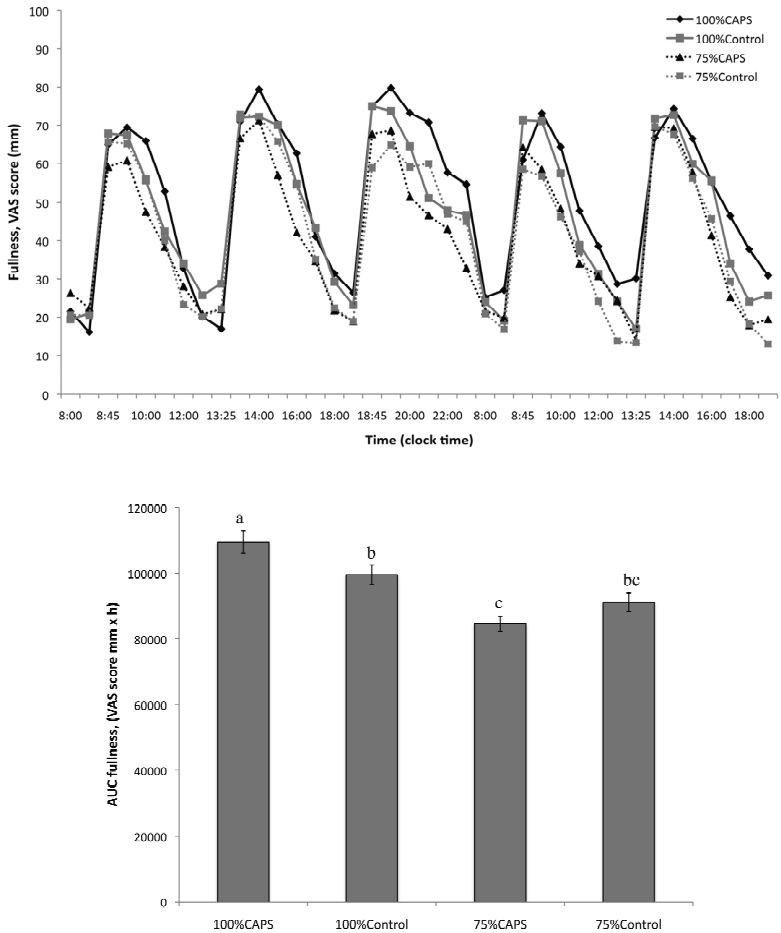

Figure 3.1 The top figure shows the changes in fullness (mm, visual analogue scale; VAS), over 36h, and interpolated between 23:00h on the first day and 8:00h on the second day, in the conditions 100%CAPS (—◆—), 100%Control (—■—), 75%CAPS (··▲··) and 75%Control (··■··). There was a trend toward lower feelings of fullness in 75%Control than in 100%Control (p=0.06). Values are means (*n*=15). The bottom figure shows the areas under the curve (AUC) for fullness over 36h, in the four conditions. Values are means, with standard errors represented by vertical bars. a – c Mean values within a row with unlike superscript letters were significantly different, p<0.05.

Pleasantness of taste, pungency, spiciness and heat sensation.

Mean pleasantness of taste ratings of the test meals were 58.3±10.4 mm for the 100%CAPS condition, 63.0±6.2 mm for the 100%Control condition, 50.7.3±12.7 mm

for the 75%CAPS condition, and 60.3±7.2 mm for the 75%Control condition. Pleasantness of taste of the test meals did not significantly differ between 100%Control and 100%CAPS nor between 100%Control and 75%Control. However, pleasantness of taste was significantly lower after 75%CAPS than after 75%Control ($p=0.01$), and significantly higher after 100%Control than after 75%CAPS ($p<0.01$). Mean ratings concerning spiciness of the meals were 67.9±3.1 mm for the 100%CAPS condition and 64.8±4.0 mm for the 75%CAPS condition, these scores represent a very spicy solution, but not too spicy (3). Mean pungency ratings were 65.7±5.9 mm for the 100%CAPS condition, 6.8±2.0 mm for the 100%Control condition, 65.3±4.7 mm for the 75%CAPS condition and 8.7±2.0 mm for the 75%Control condition ($p<0.001$). Subjects also rated whether they got a heat sensation from eating the test meals, the mean ratings concerning the heat sensation of the meals were 41.3±2.7 mm for the 100%CAPS condition, 9.9±3.0 mm for the 100%Control condition, 35.0±4.6 mm for the 75%CAPS condition and 13.8±4.5 mm for the 75%Control condition ($p<0.001$). Water intake did not differ between conditions.

Ad libitum meal

Ad libitum intake

There was a trend toward a decrease in *ad libitum* intake in the 100%CAPS condition compared with the 100%Control condition (**Figure 3.2**), subjects ate 30% less of the *ad libitum* dinner in the 100%CAPS condition compared with the 100%Control condition ($p=0.07$). They consumed 1.4±0.4 MJ in the 100%CAPS condition and 2.0±0.4 MJ in the 100%Control condition. However, this trend toward a decrease in *ad libitum* intake after addition of CAPS to the diet was not seen when subjects were fed in negative energy balance (75%CAPS, 2.4±0.4 MJ and 75%Control 2.5±0.3 MJ).
Furthermore*, ad libitum* intake was not significantly different between 100%Control and 75%CAPS nor between 100%Control and 75%Control.

Overconsumption and overcompensation

There was a trend toward decreased overconsumption after addition of CAPS to the diet in the 100% conditions ($p=0.06$) (**Table 3.3a**). Overcompensation (**Table 3.3b**) was not significantly different between 75%CAPS and 75%Control.

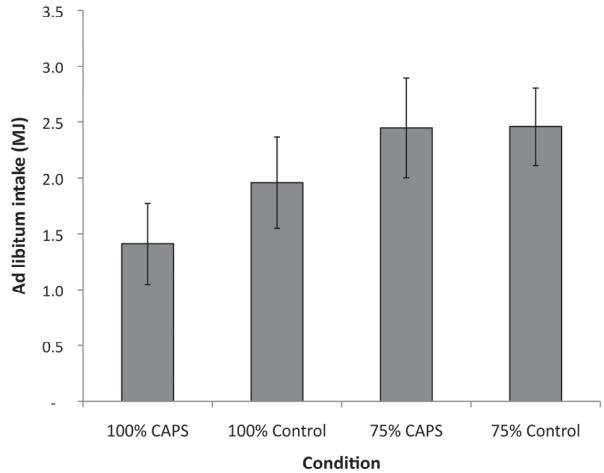

Figure 3.2 *Ad libitum* intake for the 100%CAPS, 100%Control, 75%CAPS and 75%Control conditions in 15 subjects (seven women and eight men). Values are means, with standard errors represented by vertical bars. There was a trend toward a decrease in *ad libitum* intake in the 100%CAPS condition compared with the 100%Control condition (p=0.07).

Table 3.3 Overconsumption and overcompensation (*n*=15).

A. Overconsumption

	Energy intake dinner (MJ)	Energy intake *ad libitum* dinner (MJ)	Overconsumption at dinner (%)[1]
100%CAPS	3.35 ± 0.1	4.76 ± 0.5	39.6 ± 9.4
100%Control	3.35 ± 0.1	5.30 ± 0.5	55.3 ± 10.6

Values are means with their standard errors (SE), n=15.
[1] *Ad libitum* intake expressed as % of 100% of daily energy requirement.

B. Overcompensation

	Energy intake dinner (MJ)	Energy intake *ad libitum* dinner (MJ)	Overcompensation (MJ)	Overcompensation (%)
75%CAPS	2.51 ± 0.1	4.96 ± 0.5	1.61 ± 0.4	46.4 ± 12.2
75%Control	2.51 ± 0.1	4.97 ± 0.4	1.62 ± 0.3	46.8 ± 7.8

Values are means with their standard errors (SE), n=15.

Discussion

In the present study we investigated the 36h effects of 2.56 mg CAPS (1.03 g of red chili pepper, 39,050 SHU) with every meal on appetite profile and *ad libitum* energy intake in Caucasians, in energy balance as well as in negative energy balance. We

showed that CAPS increased feelings of satiety and fullness in energy balance. Furthermore, there was a trend toward a decrease in *ad libitum* intake after addition of CAPS to the diet in the 100% conditions. After dinner, the effects of CAPS vs. Control in 25% negative energy balance prevented the effects of the negative energy balance on appetite profile compared with 100% energy intake without CAPS.

The present study supports the reported enhancing anorexigenic sensations of CAPS in energy balance. However, CAPS did not affect feelings of satiety and fullness in negative energy balance. Two previous studies investigated the effects of CAPS on appetite profile in negative energy balance (6,23). Reinbach *et al.* (23) investigated the effects of a one-day exposure to CAPS on appetite and energy intake during negative energy balance. Furthermore, they reported that CAPS relatively increased satiety and fullness during negative energy balance. Furthermore they found that a combination of CAPS and green tea suppressed hunger and increased satiety more during negative than during positive energy balance. This study lasted for three weeks, which obviously was necessary to establish this condition. An important difference between the current study and the study of Reinbach *et al*. (23) is that we tested the effect of CAPS in energy balance and in negative energy balance, confirmed by measuring the difference between energy intake and energy expenditure. Furthermore, in a previous study we investigated whether an 80% energy requirement diet of protein partly replacing carbohydrate, plus addition of CAPS, reached the same level of fullness ratings as a 100% of the energy requirement diet without high protein content, and without addition of CAPS. We did observe an increase in feelings of fullness with addition of CAPS to a diet with high protein content and we also found an increase in fullness when CAPS was added to a diet with normal protein content (6). Furthermore, in the present study we found a small but surprising effect of CAPS on desire to eat and a trend toward significance on feelings of satiety and fullness in negative energy balance. The effect had to be built up during the day, and was not acutely present after breakfast or lunch. The observation on VAS after dinner underscores our hypothesis that CAPS in 25% negative energy balance prevents the effect of the negative energy balance compared with 100% energy intake without CAPS.

In line with a previous study on the effect of CAPS on *ad libitum* intake (2), we observed a trend toward a decrease in *ad libitum* intake after addition of CAPS. However, this decrease was not seen when subjects were fed in negative energy balance. These results on the effect of CAPS on *ad libitum* intake in negative energy balance are consistent with the findings of the study by Reinbach *et al.* (23) with capsiate. Yet, capsiate was found to decrease the energy intake during positive energy balance. The present results of CAPS on *ad libitum* intake in energy balance showed that CAPS might contribute to prevention of overeating. We found a trend toward a decrease in *ad libitum* intake when CAPS was added to meals with a normal fat

content of 30 En%. This implies that the effect of CAPS on energy intake depends on the macronutrient composition of the meals: this beneficial effect of CAPS mainly has been observed with addition of CAPS to a diet with a high protein or high fat content (4,6). Although in the present study the effects of CAPS on *ad libitum* intake were smaller, there still is a trend toward significance when CAPS is added to a diet with a normal protein, normal fat content.

The addition of CAPS to the meals that were served in energy balance, did not decrease the pleasantness of taste of the meals; therefore it is not possible that the increased feelings of satiety and fullness and the trend toward decreased *ad libitum* intake in the 100%CAPS condition were due to the pleasantness of taste of the meals. The higher fullness and satiety observed with CAPS in energy balance might be supported by the higher pungency observed. Strengths of this study are the fully controlled design, measurements in energy balance as well as in negative energy balance, and knowing the magnitude of the negative energy balance. In summary, the effects of CAPS are dependent on the dosage of CAPS given, energy intake, macronutrient composition of the meals and the maximum tolerable dose of the subjects.

Taken together, the present study shows that in energy balance, addition of 1.03 g of red chili pepper to meals with a normal fat and normal protein content increases satiety and fullness, and tends to prevent overeating in Caucasians. After dinner, capsaicin prevents the effects of the negative energy balance on desire to eat.

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Chapter 4

Addition of capsaicin and exchange of carbohydrate with protein counteract energy intake restriction effects on fullness and energy expenditure

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Abstract

Introduction
Energy intake restriction causes a yo-yo effect by decreasing energy expenditure and decreasing fullness.

Objective
We investigated the 24 h effect of protein and capsaicin, singly or combined, on fullness and energy expenditure during 20% energy intake restriction.

Methods
24 subjects (12 males, 12 females, BMI: 25.2±0.4kg/m^2, age: 27±4 y; body fat: 25.6±5.7%; TFEQ: F1:6±2, F2:4±2, F3:3±2) underwent eight 36h sessions in a respiration chamber. The study had a randomized crossover design with eight randomly sequenced conditions. Subjects were fed 100% or 80% of their daily energy requirements. There were two control (C) conditions; 100%C; 80%C, two conditions with capsaicin (Caps): 100%Caps; 80%Caps, two conditions with elevated protein (P): 100%P; 80%P, and two conditions with a mixture of protein and capsaicin (PCaps): 100%PCaps; 80%PCaps. Appetite profile, EE and substrate oxidation were monitored.

Results
Compared to 100%C, 80%C showed expected negative energy-balance effects with respect to total energy expenditure, diet-induced thermogenesis and fullness, while 80%Caps counteracted these effects, and 80%P and 80%PCaps exceeded these effects ($p<0.01$). In energy balance and in negative energy balance fat-balance was more negative with 80%Caps, P, and PCaps, than with 80%C ($p<0.01$; $p<0.05$) and respiratory quotient values were lower. A negative protein-balance was prevented with 80%P and 80%PCaps vs. 80%C.

Conclusions
Results suggest that protein and capsaicin consumed singly or mixed, counteracted energy intake restriction effects on fullness and EE. During energy restriction protein and capsaicin promoted a negative fat-balance, while protein-treatments also prevented a negative protein balance.

Introduction

Obesity is associated with an increased risk for chronic diseases, including type 2 diabetes, cancer, and cardiovascular disease (1). Modest weight loss (5–10% of body weight) is associated with clinical improvements, such as decreased risk for diabetes (2), and reductions in dyslipidemia and hypertension (3). Under normal circumstances, body weight is very tightly regulated. After a period of energy intake restriction and weight loss, the body responds with several mechanisms such as increasing hunger and decreasing energy expenditure (EE) to regain its initial body weight (BW) (4). These counteractive mechanisms make adherence to weight-loss diets difficult and uncomfortable. Therefore, it is of importance to test concepts that tackle the undesirable physiological responses of the body on body-weight loss. In that perspective, several food components have been studied for their effect on appetite related feelings and thermogenesis. High-protein foods are known to have a higher thermogenic effect and to be more satiating than normal protein foods, over the short as well as over the long-term (5-7).
The protein-induced satiety may be due to elevated, (especially ketogenic), plasma amino acid concentrations, diet-induced thermogenesis, hunger suppression, and possibly increased anorexic hormone concentrations (8). The protein-induced energy-expenditure may be due to protein–related high diet-induced thermogenesis of 20-30%, due to protein turn-over, urea production, and gluconeogenesis (8). Moreover, a protein-diet stimulates fat-oxidation (5,6,8), partly due to the higher energy-expenditure leading to faster glycogen depletion especially overnight and thus a larger fat oxidation. Then mRNA levels of genes involved in carbohydrate and lipid metabolism in the liver are affected. In liver, a protein diet decreases mRNA encoding glycolysis enzymes (glucokinase, L-pyruvate kinase) and lipogenesis enzymes (acetyl-CoA carboxylase, fatty acid synthase), increases mRNA encoding gluconeogenesis enzymes (phosphoenolpyruvate carboxykinase), first lowers, then restores mRNA encoding glycogen synthesis enzyme (glycogen synthase), and does not change mRNA encoding β-oxidation enzymes (carnitine palmitoyltransferase 1, peroxisomal acyl-coenzyme A oxidase 1, beta hydroxyacyl CoA dehydrogenase) (9). Following a protein diet, carbohydrate oxidation is increased above carbohydrate intake resulting in a negative carbohydrate balance while lipogenesis is decreased explaining the negative fat balance in protein diets (5,6,8,9).

Capsaicin, the pungent ingredient of red pepper, has been reported to increase EE and DIT (diet-induced thermogenesis), probably due to ß-adrenergic stimulation, and to decrease the respiratory quotient (RQ), implying a shift in substrate oxidation from carbohydrate to fat oxidation (10-13). The addition of capsaicin to a diet has also been shown to increase satiety and decrease appetite and cumulative food intake (13-15). Whether, in negative energy balance, during weight-reduction, reductions in EE and

feelings of fullness can be prevented by consuming protein and capsaicin remains to be shown. Therefore, the aim of the present study was to investigate whether an 80% energy requirement diet consisting of partly replacing carbohydrate by protein, plus addition of capsaicin reaches the same level of fullness ratings and EE as a 100% energy requirement control diet. To proof this concept, studies in subjects in energy-balance (100%) as well in negative energy-balance (80%), consuming control diets (100%C and 80%C), control diets with added capsaicin (100%Caps and 80%Caps), diets with carbohydrate replaced in part by protein (100%P and 80%P), and diets with added capsaicin and with carbohydrate replaced in part by protein (100%PCaps and 80%PCaps) were performed over two days in a fully energy-balance controlled condition in the respiratory chamber.

Subjects and methods

Subjects

Twenty-eight healthy subjects were recruited by advertisements in the local newspapers and on notice-boards of the university. Subjects were selected based on age, height, weight and BMI. The power calculation was based upon values of hunger and energy expenditure and its components from studies by Hochstenstein-Waelen *et al*. (16,17), by Lejeune *et al*. (14) and by Westerterp-Plantenga *et al*. (18). Eating behavior was assessed using a validated Dutch translation of the Three Factor Eating Questionnaire (TFEQ) (19). Cognitive restrained and unrestrained eating behavior (Factor 1), emotional eating and disinhibition (Factor 2), and the subjective feeling of hunger (Factor 3) were scored. Cognitive restraint (F1) implies full control over the amount and type of food intake; disinhibition (F2) implies inhibition of cognitive restraint, as well as emotional eating; the factor hunger implies a continuous feeling of hunger (F3) (19). Subjects were excluded from participation if their factor 1 score on the TFEQ was >9. Subjects mean score on the TFEQ was F1: 6±2, F2: 4±2 and F3: 3±2, indicating low levels of cognitive restraint, disinhibition, and general hunger. Selected subjects were healthy, not taking medication, non-smoking and not dieting; they gave written informed consent and the study was approved by the Medical Ethics Committee of Maastricht University. The study was conducted in de metabolic unit of Maastricht University, department of Human Biology.

Study protocol

To test the hypothesis that feeding the subjects 80% of their individual energy requirement with addition of capsaicin and with partly and iso-energetically replacing

carbohydrate by protein, would preserve energy expenditure and fullness, the following assumptions had to be confirmed:
(I) Achievement of negative energy balance as a result of feeding 80% of energy requirement (80%C).
(II) Decreased fullness and decreased EE in the 80% energy requirement control condition (80%C).
(III) Increased EE and fullness in energy balance with added capsaicin and partly and iso-energetically replacing carbohydrate by protein (100%PCaps) as well as with only capsaicin addition (100%Caps), and with only carbohydrate replacement by protein (100%P).
The eight conditions (100%C, 80%C, 100%Caps, 80%Caps, 100%P, 80%P, 100%PCaps, 80%PCaps) had a single-blind randomized crossover design. The respiratory chamber sessions were conducted four weeks apart in women to ensure that each female subject was in the same phase of her menstrual cycle. In men, the sessions were conducted at least seven days apart. Two days prior to each session, subjects were provided with a standardized diet to consume at home in order to be fed in energy balance, and to receive the same macronutrient proportions as during the respiration chamber experiment. Moreover, caffeine intake was standardized at a maximum of 100 mg/day (one cup of coffee, or three cups of tea). Before the test was started, subjects were asked whether they had encountered any difficulties while consuming the diet at home.
For the respiratory chamber measurements, the subjects entered the respiration chamber at 8:00 hrs after an overnight fast. Subjects were instructed to go to bed around 23:00 hrs. The next day, subjects followed the same protocol as the day before. At 8:00 hrs on the second day the subjects were released from the respiration chamber.

Experimental diets

The energy content of the diet that the subjects consumed at home was based on Basal Metabolic Rate (BMR) calculated with the equation of Harris-Benedict (20), and multiplied by an activity index of 1.7 (21). In the respiration chamber energy requirements were calculated based calculated BMR multiplied by an activity index of 1.4. Subjects had to completely finish all drinks and meals. For the presentation of the control and treatment conditions for food and energy intake, see **Table 4.1.**

Appetite profile

Appetite profile (hunger, fullness, satiety and desire to eat) was measured using anchored 100-mm visual analogue scales (VAS). During each 36 h session these questionnaires were completed every waking hour, and before and after every meal.

The scale is anchored from 'not at all' on the left to 'extremely' on the right. The VAS data are given as area under the curve (AUC). Area under the curve is the area above the baseline, calculated by the conventional trapezoid rule.

Table 4.1 Control and treatment conditions for food- and energy intake in the respiratory chamber.

Conditions	C (100 and 80%)	Caps (100 and 80%)	P (100 and 80%)	PCaps (100 and 80%)
Macronutrient composition protein/fat/carbohydrate (En%)	10/30/60	10/30/60	25/30/45	25/30/45
Addition of red pepper (mg)	0	1030	0	1030
Capsules/meal	2 placebo	2 red pepper	2 placebo	2 red pepper
Meals				
Breakfast (15%EI)	Coconut bread and orange juice	Coconut bread and orange juice	Bread and cheese and milk	Bread and cheese and milk
Lunch (35%EI)	Pasta, tomato-sauce, sausages, and a cucumber-tomato salad	Pasta, tomato-sauce, sausages, and a cucumber-tomato salad	Pasta, tomato-sauce, sausages, and a cucumber-tomato salad	Pasta, tomato-sauce, sausages, and a cucumber-tomato salad
Dinner (50%EI)	Pasta, tomato-sauce, sausages, fruit	Pasta, tomato-sauce, sausages, fruit	Pasta, tomato-sauce, sausages, yoghurt	Pasta, tomato-sauce, sausages, yoghurt
Water and decaffeinated coffee	*Ad libitum*	*Ad libitum*	*Ad libitum*	*Ad libitum*

[1] C: control; Caps: capsaicin; P: protein; PCaps: protein plus capsaicin, *n* = 24. Red pepper: Cayenne, Solaray, Park City, UT, USA 40,000 Scoville Heat Units. For 100%energy balance, energy intake is based upon energy requirement, calculated as 1.4 x BMR. BMR is calculated following Harris & Benedict (20).

Anthropometrics

Anthropometric measurements were performed at baseline. Subjects were weighed in their underwear after an overnight fast, using a calibrated hospital scale to the nearest 0.1 kg (Tanita TBF-310). Height was measured at screening to the nearest 0.1 cm (Seca-stadiometer). Body composition was measured by using the deuterium dilution technique. $^{2}H_2O$ dilution was used to measure total body water (TBW). The subjects were asked to collect a urine sample in the evening just before drinking the deuterium-enriched water solution. After ingestion of this solution, the subjects went to bed and no additional consumption was allowed for this period. Ten hours after drinking the water solution, another urine sample was collected. The dilution of the deuterium isotope is a measure of the TBW of the subject. Deuterium was measured in the urine samples with an isotope ratio mass spectrometer (VG-Isogas Aqua Sira; VG Isogas, Middlewich, England). TBW was obtained by dividing the measured deuterium dilution space by 1.04. Fat-free mass (FFM) was calculated by dividing TBW by the hydration factor 0.73. Fat mass (FM) was determined as BW-FFM (7,22,23).

Indirect calorimetry

Oxygen consumption and carbon dioxide production were measured in the respiration chamber (14,24). The respiration chamber is a 14-m^3 room furnished with a bed, chair, computer, television, radio cassette player, telephone, intercom, sink, and toilet. The room was ventilated with fresh air at a rate of 70-80 L/min. The ventilation rate was measured with a dry gas meter (type 4; Schlumberger, Dordrecht, Netherlands). The concentrations of oxygen and carbon dioxide were measured with the use of an infrared carbon dioxide analyzer (Uras 3G; Hartmann and Braun, Frankfurt, Germany) and 2 paramagnetic oxygen analyzers: Magnos 6G (Hartmann and Braun) and type OA184A (Servomex, Crowborough, United Kingdom). During each 15-min period, 6 samples of outgoing air for each chamber, 1 sample of fresh air, zero gas, and calibration gas were measured. The gas samples to be measured were selected by a computer that also stored and processed the data (24).

Energy expenditure and substrate oxidation

Twenty-four–hour EE, as measured in the respiratory chamber, consists of sleeping metabolic rate (SMR), diet induced thermogenesis (DIT), and activity-induced energy expenditure (AEE). Activity was monitored with a radar system based on the Doppler principle. SMR was defined as the lowest mean EE measured over 3 consecutive hours between 00:00 and 07:00 AM. REE was calculated by plotting EE against radar output; both were averaged over 30-min periods. The intercept of the regression line at the lowest radar output represents the energy expenditure in the inactive state (resting energy expenditure; REE), which consists of SMR and DIT (24). DIT was determined by subtracting SMR from REE. AEE was determined by subtracting SMR and DIT from 24 h EE. Carbohydrate, fat, and protein oxidation were calculated from the measurements of oxygen consumption, carbon dioxide production, and urinary nitrogen excretion by using the formula of Carpenter in Brouwer *et al*. (25). Urine samples were collected from the second void on the day subjects entered the respiration chamber to 17:00 on the next day. Samples were collected in containers with 10 mL H_2SO_4 to prevent nitrogen loss through evaporation. Volume and nitrogen concentration were measured, the latter with a nitrogen analyzer (CHN-O-Rapid; Heraeus, Hanau, Germany).

Statistical analyses

Statistical tests were performed using Statview SE Graphics software (version 4.5; Abacus Concepts Inc, Berkeley, CA, USA). Factor ANOVA repeated measures with Bonferroni corrections were performed to determine possible differences in energy expenditure, substrate oxidation and appetite profile between the eight conditions. Specific comparisons were made using Scheffé's F. ANOVA repeated measures with

gender as covariate was used to determine possible differences between male and female subjects. All statistical tests are two-sided and differences are considered statistical significant if $p<0.05$. Data are presented as means and standard deviations unless otherwise indicated.

Results

Twenty-four healthy subjects (12 males and 12 females) completed the eight conditions. The subjects had a mean age of 27±4 y, BMI of 25.2±0.4kg/m^2 and body fat of 25.6±5.7%. Subjects mean score on the TFEQ was F1: 6±2, F2: 4±2 and F3: 3±2, indicating low levels of cognitive restraint, disinhibition, and general hunger. No adverse events occurred. For the results no differences from the interventions between men and women appeared, therefore the results were taken together (**Table 4.2**).

Respiratory chamber experiments

Energy balance was achieved in the 100% control condition. In the 100% condition with P or PCaps, energy balance was slightly but significantly negative, due to the elevated (total energy expenditure) TEE, while there was no difference in EI. In the 80% control condition, negative energy balance was achieved. When the subjects consumed 80% of their individual energy requirements, negative energy balance was -1.6±0.2 MJ, due to the reduced energy intake minus the significant energy expenditure reductions in SMR and DIT, adapting to the reduced energy intake. In the 80% condition with Caps, P, and PCaps, negative energy balance was significantly more negative than in the 80% control condition, due to a higher SMR and DIT, resulting in a higher TEE than in the 80% control condition (**Table 4.2**). The following comparisons are relevant (see also **Table 4.2**). When the subjects were fed in energy balance, TEE with Caps ($p<0.05$), P ($p<0.01$), and PCaps ($p<0.01$) was higher than TEE with 100%C. In the 80% energy intake condition, TEE was higher with Caps ($p<0.05$), P ($p<0.05$), and PCaps ($p<0.01$), than TEE with 80%C. At 80% energy intake, TEE with 80%Caps did not differ significantly from TEE at 100%C, while TEE was, as expected, lower for 80%C; with P ($p<0.05$) and with PCaps ($p<0.05$) at 80% energy intake, TEE was higher than with 100%C. TEE was, as expected, lower for 80%Caps vs. 100%Caps, 80%P vs. 100%P, 80%PCaps vs. 100%PCaps ($p<0.01$).
Elevated TEE with Caps, protein, or PCaps treatments consisted of elevated DIT, while SMR or AEE did not differ. Reduced TEE in the 80%C vs. 100%C condition consisted of reduced DIT and sometimes reduced SMR, while AEE did not differ. Reduced TEE in the 80%Caps vs. 100% Caps, 80%P vs. 100%P, 80%PCaps vs. 100%PCaps conditions consisted of reduced DIT, while SMR and AEE had remained the same **(Table 4.2).**

Table 4.2 Total energy expenditure, components of energy expenditure, energy intake, RQ, substrate oxidation and fullness scores during the eight conditions: 100%C, 80%C, 100%Caps, 80%Caps, 100%P, 80%P 100%PCaps and 80%PCaps.

	100%C	80%C	100%Caps	80%Caps	100%P	80%P	100%PCaps	80%PCaps
TEE *(MJ/d)**	10.1±0.2^{a}	9.6±0.2^{b}	10.3±0.2^{c}	10.0±0.2^{a}	10.4±0.2^{c}	10.2±0.2^{d}	10.6±0.2^{e}	10.3±0.2^{c}
TEE *% of 100%C*	100	95	102	100	103	101	105	102
SMR *(MJ/d)**	7.2±0.2^{a}	6.9±0.2^{b}	7.1±0.2^{a}	7.1±0.2^{a}	7.1±0.2^{a}	7.1±0.2^{a}	7.1±0.2^{a}	7.1±0.2^{a}
DIT *(MJ/d)**	1.0±0.1^{a}	0.8±0.1^{b}	1.3±0.1^{c}	1.0±0.1^{a}	1.4±0.1^{c,d}	1.2±0.1^{c}	1.6±0.1^{d}	1.3±0.1^{c}
DIT *% of EI*	9.9	10.0	12.9	12.5	14.9	15.0	15.8	16.3
AEE *(MJ/d)*	1.9±0.1	1.9±0.1	1.9±0.1	1.9±0.1	1.9±0.1	1.9±0.1	1.9±0.1	1.9±0.1
EI *(MJ/d)**	10.1±0.4^{a}	8.0±0.4^{b}	10.1±0.4^{a}	8.0±0.4^{b}	10.1±0.4^{a}	8.0±0.4^{b}	10.1±0.4^{a}	8.0±0.4^{b}
EB *(MJ/d)**	0.0±0.2^{a}	-1.6±0.2^{b}	-0.2±0.2^{c}	-2.0±0.2^{d}	-0.3±0.2^{c}	-2.1±0.2^{d}	-0.5±0.2^{e}	-2.3±0.2^{d}
EB *% of 100% EB*	100	-16	-2	-20	-3	-21	-5	-23
RQ*	0.89±0.01^{a}	0.87±0.01^{b}	0.88±0.01^{c}	0.86±0.01^{d}	0.87±0.01^{b}	0.86±0.01^{d}	0.86±0.01^{d}	0.85±0.01^{e}
RQ *% of 100%C*	100	98	99	97	98	97	97	96
Fat balance *(g/d)**	2±1^{a}	-14±6^{b}	-8±4^{c}	-19±6^{d}	-10±5^{b,c}	-20±6^{d}	-12±6^{b}	-22±5^{d}
Carbohydrate balance *(g/d)**	-4±1^{a}	-53±19^{b}	6±6^{c}	-72±18^{d}	-38±15^{e}	-78±23^{d}	-45±17^{b,e}	-85±29^{d,f}
Protein balance *(g/d)**	-1±4^{a}	-10±4^{b}	0±2^{a}	-8±4^{b}	42±6^{c}	-1±3^{a}	42±6^{c}	-1±3^{a}
Fullness scores *(AUC)**	1440±98^{a}	1224±112^{b}	1584±102^{c}	1421±112^{a}	1721±147^{d}	1593±121^{c}	1839±132^{e}	1664±118^{c,d}
Fullness *% of 100%C*	100	85	110	99	120	110	128	115

[1] Values are mean ± SEM, *n* = 24. Data were analyzed using ANOVA repeated measures. *Overall effects between the eight conditions; p<0.001. Means in a row without a common letter differ, p<0.05. AEE: Activity-induced energy expenditure; DIT: Diet-induced thermogenesis; EB: Energy balance; EI: Energy intake; RQ: Respiratory quotient; SMR: Sleeping metabolic rate; TEE: Total energy expenditure. In the following conditions: Control: 100%C and 80%C (% energy from carbohydrate/protein/fat: 60/10/30); with P or with PCaps: (% energy from carbohydrate/protein/fat : 45/25/30), with capsaicin: 1030 mg red pepper/ meal; with Caps: C plus 1030 mg red pepper/meal. Magnitudes of differences are expressed in %.

Substrate oxidation

RQ was significantly lower, and fat-balance was more negative, in the 100%Caps ($p<0.05$), P and PCaps ($p<0.01$) conditions than in the 100% control condition.
Similarly, RQ was significantly lower, and fat-balance was more negative, in the 80%Caps ($p<0.05$), P ($p<0.05$), and PCaps ($p<0.01$) conditions than in the 80% control condition. In the P and PCaps conditions protein balances were more positive than in the similar 80% or 100% control or Caps conditions (**Table 4.2**).

Fullness

Since the ratings of fullness and of feelings of satiety were similar, and since these ratings were opposite to the ratings of hunger and desire to eat, only the ratings of fullness are shown. Comparing the AUC over 24 h of fullness with the control conditions, it appeared that fullness was higher in the 100%Caps ($p<0.05$), 100%P ($p<0.01$), and 100%PCaps ($p<0.01$) groups than in the 100%C group. Also fullness was higher in the 80%Caps ($p<0.05$), 80%P ($p<0.01$) and 80%PCaps ($p<0,01$) groups than in the 80%C group. Fullness was higher in the 80%P and 80%PCaps groups than in the 100%C group ($p<0.05$), whereas it was lower in the 80%C group than in the 100%C group ($p<0.01$) (**Table 4.2**).

Discussion

In the present study, the central question was whether in negative energy balance, the original level of EE and fullness as in neutral balance, is preserved, when capsaicin is added to the diet and carbohydrate is partly replaced by protein. To test the hypothesis that feeding the subjects 80% of their individual energy requirement with addition of capsaicin and with partly and iso-energetically replacing carbohydrate by protein, would preserve energy expenditure and fullness, the following assumptions were made, and had to be confirmed: (I) Achievement of negative energy balance as a result of feeding 80% of energy requirement (80%C); (II) Decreased fullness and decreased EE in the 80% energy requirement control condition (80%C). (III) Increased EE and fullness in energy balance with added capsaicin and partly and iso-energetically replacing carbohydrate by protein (100%PCaps) as well as with only capsaicin addition (100%Caps), and with only carbohydrate replacement by protein (100%P). All assumptions were confirmed, in line with previous studies, which assessed administration of these ingredients individually (5, 6, 10-15, 18, 26-37) as well as in combination (38,39). The short-term controlled studies assessed these ingredients mainly in energy balance (6,9-12,14-17,26,28,30,33,37), while longer-term studies assessed these effects in negative energy balance (5,13,27,29,34-36). The

present studie shows the effects of these ingredients measured over 24 h under controlled energy and negative energy balance conditions.

Negative energy balance was achieved by feeding the subjects 80% of their energy requirements. It then appeared that the 20% underfeeding did not result in a 20% negative energy balance, yet, due to physiological adaptation of DIT and SMR, it resulted in a 16% negative energy balance in the control condition. In the treatment conditions of Caps, P, or PCaps it resulted in a 20-23% negative energy balance condition, because a possible reduction in SMR was prevented and DIT was increased compared to the 100%C condition, resulting in a similar TEE with Caps, and in an increase in TEE with P, and PCaps. With respect to energy expenditure, the key components that prevent TEE to decrease in negative energy balance or even increase TEE, are DIT and SMR. The observation that in energy balance DIT increases upon addition of capsaicin or exchange of carbohydrate with protein has been shown before (10-13,30,33); a relative increase in SMR in energy balance has also been shown before (6,15,16). We speculated that the acute increase in DIT (30) might over a few days develop in an increase in SMR (13,15,16), while DIT even may rebound (15,16). This increase in SMR may be an adaptation of the body to a high-protein diet with respect to an enhanced protein turn-over, and a positive nitrogen balance. Enhanced protein-turn-over with a higher protein-diet has been observed in rats, but it has not yet been confirmed in humans (31). Moreover, in the 80% energy intake control condition fullness was decreased with 15%, while with 80% Caps condition fullness did not differ from 100% C, and in the 80% condition with the treatments P and PCaps, fullness was increased with 10-15% compared to the 100% control condition.

Answering the main question did not only reveal that addition of capsaicin and application of partly iso-energetically carbohydrate replaced by protein would prevent the usual reduction in fullness and EE, but in the conditions with exchange of carbohydrate with protein, it even exceeded it. Obviously, the magnitudes of the differences were that powerful, that a surplus occurred. These data suggest that reducing effects of energy restriction on EE may be prevented or reversed by capsaicin, protein and by capsaicin plus protein. Replacing carbohydrate with protein shows the largest effect. Effects of this treatment did not differ significantly from the same treatment with capsaicin added to it. Adding capsaicin only slightly increased the effect, not reaching statistical significance ($0.05<p\leq0.10$). Nevertheless, it was shown that capsaicin was able to counteract 20% energy intake reductions effects, namely EE and fullness.

Administration of protein and of protein plus capsaicin during energy restriction in the present study resulted furthermore in lowering of the fat balance and preservation of the protein balance. The exchange of 15% of total energy intake from carbohydrate for protein and addition of capsaicin given during energy restriction prevented the 24 h protein balance from becoming negative. None of the observed effects was

influenced by differences in activity, as there were no differences in AEE between the four conditions. Furthermore, the capsaicin alone, protein alone, and protein plus capsaicin were well tolerated by all subjects. We observed an immediate negative effect on TEE of energy restriction when comparing TEE in condition 80%C with TEE in condition 100%C. This decline in TEE due to energy restriction was however completely abolished with just capsaicin, and even over-compensated by protein and by protein plus capsaicin. Here we report the effects over a 2-day period. We do not know whether the observed effects persist over time or whether compensatory mechanisms will be triggered. In a previous weight-loss study the additional thermogenic effect of a bioactive supplement was sustained over 8 weeks, which suggests that stimulating effects of bioactive components on EE do not diminish over time (38). The results are in agreement with previous studies comparing administration of these ingredients individually (5,6,10-15,18,26-37) and in combination (38,39). Despite the main limitation of the study namely that it was conducted over a relatively short period of time, results are in line with those from previous studies, which showed that an adaptation of 2 days before to the macronutrient composition offered is sufficient (6,37). Although subjects were not adapted to 20% energy-restriction, nor to receive the additional capsaicin before the corresponding test periods it is unlikely that the effects that were obtained would only be transient. Previous studies reported that both capsaicin addition (13) and a carbohydrate-protein exchange (5) during 3 months negative energy balance, still showed a higher satiety compared to baseline, and a prevention of a decrease in resting energy expenditure (5,13). In those studies, due to the weight loss, the increased energy expenditure as was shown in the present study, was at least sustained as a prevention of the usual decrease (5,13). The novelty of this study is the comparison of the single as well as the combined treatments, and the comparisons of the magnitudes of the effects. Moreover, the study proposes to follow the paradigm of introducing a negative energy balance and offer ingredients to be able to support this, instead of offering ingredients and waiting until a negative energy balance would occur spontaneously. Furthermore, the study shows that when the magnitudes of effects of ingredients on TEE in energy balance are ~2-5%, counterbalancing in negative energy balance may be expected. With respect to the appetite profile, when the effects on fullness and related questions are 10-30% in energy balance, an effect in negative energy balance still is likely to occur.

In summary, a combination of addition of capsaicin, and carbohydrate replacement by protein with a 20% energy restricted diet, or carbohydrate/protein exchange alone resulted in higher EE and fullness compared to a control diet in energy balance. Fat balance was more negative in the capsaicin addition plus carbohydrate/protein exchange vs. control energy-restricted diet. Therefore, a combination of protein and capsaicin, or capsaicin or protein alone, may at least maintain normal levels of EE and fullness during energy restriction.

The effectiveness of the capsaicin and protein should be further evaluated in well-designed weight-loss studies in overweight and obese subjects.

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Chapter 5

The role of catechol-O-methyl transferase *Val(108/158)Met* polymorphism (rs4680) in the effect of green tea on resting energy expenditure and fat oxidation

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Abstract

Introduction
Green tea (GT) is able to increase energy expenditure (EE) and fat oxidation (FATox) via inhibition of catechol-O-methyl transferase (COMT) by catechins. However, this does not always appear unanimously because of large inter-individual variability. This may be explained by different alleles of the functional COMT *Val108/158Met* polymorphism that are associated with COMT enzyme activity; high-activity enzyme, $COMT^{H}$ (*Val/Val* genotype), and low-activity $COMT^{L}$ (*Met/Met* genotype).

Methods
Fourteen Caucasian subjects (BMI: 22.2±2.3 kg/m^2, age: 21.4±2.2 years) of whom 7 with the $COMT^{H}$-genotype and 7 with the $COMT^{L}$-genotype were included in a randomized, cross-over study in which EE and substrate oxidation were measured with a ventilated-hood system after decaffeinated GT and placebo (PL) consumption.

Results
At baseline, EE, RQ, FATox and carbohydrate oxidation (CHOox) did not differ between groups. Significant interactions were observed between COMT genotypes and treatment for RQ, FATox and CHOox ($p<0.05$). After GT vs. PL, EE (GT: 62.2 vs. PL: 35.4 kJ.3.5hrs; $p<0.01$), RQ (GT: 0.80 vs. PL: 0.83; $p<0.01$), FATox (GT: 18.3 vs. PL: 15.3 g/d; $p<0.001$) and CHOox (GT: 18.5 vs. PL: 24.3 g/d; $p<0.001$) were significantly different for subjects carrying the $COMT^{H}$ genotype, but not for subjects carrying the $COMT^{L}$ genotype (EE, GT: 60.3 vs. PL: 51.7 kJ.3.5hrs; NS), (RQ, GT: 0.81 vs. PL: 0.81 ; NS), (FATox, GT: 17.3 vs. PL: 17.0 g/d; NS), (CHOox, GT: 22.1 vs. PL: 21.4 g/d; NS).

Conclusions
Subjects carrying the $COMT^{H}$ genotype increased energy expenditure and fat-oxidation upon ingestion of green tea catechins vs. placebo, whereas $COMT^{L}$ genotype carriers reacted similarly to GT and PL ingestion. The differences in responses were due to the different responses on PL ingestion, but similar responses to GT ingestion, pointing to different mechanisms.
The different alleles of the functional COMT *Val108/158Met* polymorphism appear to play a role in the inter-individual variability for EE and FATox after GT treatment.

Introduction

Overweight and obesity represent a rapidly growing threat to the health of populations worldwide and is caused by an imbalance between energy intake and energy expenditure (EE) (1,2). A negative energy balance is needed to produce weight loss and can be achieved by either decreasing intake or increasing expenditure (3,4). Amongst others, stimulation of EE by green tea (GT), rich in catechins and caffeine has attracted interest, especially because GT does not contain any energy itself, yet stimulates EE. Tea is made from the leaves of Camellia sinensis L. species of the Theaceae family, GT being the non-oxidized, non-fermented product, containing high quantities of several polyphenolic components such as epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate (5). Caffeine has also been shown to stimulate thermogenesis and fat oxidation in humans (6-8). The methylation of catechins by catechol-O-methyltransferase (COMT) and the inhibition of phosphodiesterase by caffeine appear to be the principal mechanisms behind the stimulating properties of GT. The importance of GT catechins in stimulating EE was shown by Dulloo *et al*. (9) who observed that the thermogenic effect of GT extract containing caffeine and catechins, is greater than that of an equivalent amount of caffeine. However, a thermogenic effect as well as a weight reducing effect has not been unanimously shown, indicating the presence of moderating factors. The difference in outcome between several ethnic populations suggests a role for genetic predisposition, which was supported by recent meta-analyses (10,11) that addressed effects of GT on weight loss and thermogenesis. Different polymorphisms for COMT enzyme activity exist and these may be responsible for the variability in flavonoid O-methylation that was previously reported by Hodgson *et al*. (12). The *Val(108/158)Met* polymorphism replaces valine by methionine, thereby changing enzyme activity. The inter-individual variability of the activity of COMT could vary as much as 3-fold. Moreover, there is evidence that there is a difference in COMT enzyme activity between ethnic groups (13). Asian populations appear to have a higher frequency of the thermostable, high activity enzyme, $COMT^{H}$ (*Val/Val* genotype) than the Caucasian populations that have a higher frequency of the thermolabile, low activity enzyme $COMT^{L}$ (*Met/Met* genotype); half of Caucasians are homozygous for $COMT^{L}$ (25%) or $COMT^{H}$ (25%). The other 50% of this population is heterozygous (*Val/Met* genotype) (13). This may explain the difference in sensitivity to interventions with GT, and why, in some studies with Caucasian subjects, no effect was seen after ingestion of GT. Hence, the aim of this pilot study was to examine the role of genetic predisposition in the effect of GT by measuring treatment induced EE and substrate oxidation after GT and placebo (PL) consumption in Caucasian subjects carrying either a $COMT^{H}$ genotype or $COMT^{L}$ genotype.

Subjects and methods

Subjects

Fourteen healthy Caucasian subjects participated in this study after recruitment by advertisements in local newspapers and on notice boards at the university. All volunteers (N=24) participated in an initial screening that involved measurements of body weight and height, blood sampling and included the completion of a questionnaire related to eating behavior (Three Factor Eating Questionnaire, TFEQ (14)) and the completion of a questionnaire related to health, use of medication, physical activity, alcohol consumption, food allergies, smoking behavior and daily caffeine consumption. All subjects were in good health, non-smokers, not using medication (except for contraception), at most moderate alcohol consumers and unrestrained eaters (as assessed by factor 1 of the TFEQ). Subject recruitment started in September 2009 and the study was conducted between February 2010 and June 2010. This study was conducted according to the guidelines of the Declaration of Helsinki and all procedures involving human subjects were approved by the Medical Ethical Committee of Maastricht University Medical Centre. Written informed consent was obtained from all subjects. The study was registered as follows: Nederlands Trial Register, registration number NTR1918.

Experimental design

The study had a randomized, two arms, single-blind, crossover design. Subjects attended the university-laboratory once a week, during two consecutive weeks. They were instructed to abstain from caffeine-rich products like tea, coffee, cola-type soft drinks and energy drinks for at least 3 days before the test day. They traveled by public transport or by car, in order to avoid physical activity that would have increased EE at rest. Subjects arrived in the fasted state at 08.15h and were kept in a time-blinded surrounding. They emptied their bladder before the test. During the test subjects were lying in the supine position. After resting on a bed for 30 minutes, the EE at rest and the substrate oxidation of the subjects was measured for 30 minutes by means of an open-circuit, ventilated-hood system. Gas analysis was performed by a paramagnetic oxygen analyzer (omnical type 1155B, Crowborough Sussex, UK) and an infrared carbon dioxide analyzer (omnical type 1520/1507). Energy expenditure was calculated using Weir's formula (15). The RQ was calculated as CO_2 produced/O_2 consumed. Fat oxidation (FATox) and carbohydrate oxidation (CHOox) were calculated in grams/3.5 hrs with the formulas of Carpenter (16).

To test the effect of GT *vs.* PL on thermogenesis, subjects ingested in random order three GT capsules (Content: Sunphenon 90 LB, Taiyo Kagaku Co. Ltd, Mie, Japan; Capsules: Gelkaps, Falkenhagen, Germany), or the control, which were three PL

capsules (Gelkaps, Falkenhagen, Germany), after measuring EE at baseline. In addition, subjects received water (100 ml) in order to swallow the capsules easily. The capsules all had the same appearance. The composition and the dose of the capsules are presented in **Table 5.1**. During the consumption of the capsules the hood was removed temporarily. After the ingestion the hood was placed back and the measurements continued for another 3.5 hours, during which the increase in thermogenesis was determined. Subjects were not allowed to talk, laugh, move or sleep while lying under the hood (17).

Table 5.1 Ingredients per capsule (mg).

	GT	PL
Total polyphenols	207.1	-
Total catechins	*169.0*	-
Epigallocatechin gallate	*84.5*	-
Caffeine	4.2	-
Soy oil	316.9	528.2
Total filling weight	528.2	528.2
Total weight capsule	757.0	757.0

PL, placebo; GT, green tea; GT: Sunphenon 90 LB (Taiyo Kagaku Co. Ltd, Mie, Japan) decaffeinated green tea extract. Subjects received three capsules per test day.

DNA isolation and genotyping

Blood was collected in an EDTA tube during screening and the buffy coat was stored at -80°C. Genomic DNA was isolated from the buffy coat using the QIAamp mini blood kit (Qiagen, Amsterdam, The Netherlands). Genotyping of the *Val(108/158)Met* polymorphism of the COMT gene (RS4680) was performed using a commercially available TaqMan SNP genotyping assay from Applied Biosystems (Foster City, California, USA). The procedure was performed according to the manufacturer's protocol and measured on an Applied Biosystems 7900 HT Fast Real-Time PCR system. Allelic calls were determined semi-automatically using the allelic discrimination software of Applied Biosystems.

Statistical analysis

Data are presented as means ± standard errors, unless otherwise indicated. A Chi-square test was used to check whether the genotype frequencies were in Hardy Weinberg equilibrium. A one-way ANOVA was used to assess possible differences with respect to subject characteristics between genotypes, at baseline. A two-factor ANOVA with genotype ($COMT^H$ vs $COMT^L$) as factor 1 and treatment (GT vs PL) as factor 2 was used to assess differences between genotype and treatment, as well as a

possible interaction effect. Pairwise comparisons allowed us to locate the significant differences between treatments or between genotypes. Also, deltas (GT-PL) were calculated to compare the relative changes between COMT genotypes, which emphasize the interaction between COMT genotypes and treatment. A one-way ANOVA was used to assess possible differences for subject characteristics between genotypes. Data were analyzed using PASW Statistics 18.0 (SPSS Inc. Chicago, Illinios, USA). The level for establishing significant differences was taken at $p<0.05$.

Results

Fourteen Caucasian subjects (8 females, 6 males) participated in this study, they were healthy, aged 19 to 27 years and had a BMI between 18 and 26 kg/m^2 **(Table 5.2)**. From the initial 24 volunteers, one COMT^H carrier withdrew from the study without giving any reason. Also, 9 subjects carried the intermediate-activity COMT^M genotype and were therefore not included in this study **(Figure 5.1)**. No adverse events occurred as no subject reported any discomfort after consuming the capsules. No different effects between men or women were observed therefore these data have been pooled together. The single nucleotide polymorphism of the COMT genotype was in Hardy Weinberg equilibrium (**Table 5.3)**.

Table 5.2 Subject characteristics

	Total	COMT^H	COMT^L	*P-values*
N (M/F)	14 (6/8)	7 (4/3)	7 (2/5)	
Age (year)	22.0 ± 2.3	22.9 ± 2.7	21.1 ± 1.5	0.193
Height (m)	1.76 ± 0.08	1.76 ± 0.09	1.75 ± 0.09	0.814
Body weight (kg)	69.3 ± 11.3	71.4 ± 7.8	67.1 ± 14.4	0.284
BMI (kg/m^2)	22.4 ± 2.6	23.0 ± 2.1	21.7 ± 3.1	0.176
FM (kg)	15.3 ± 6.5	16.8 ± 3.6	13.8 ± 8.5	0.257
FFM (kg)	54.0 ± 6.6	54.6 ± 7.5	53.3 ± 6.1	0.703

COMT^H , high activity catechol-O-methyl transferase genotype; COMT^L , low activity catechol-O-methyl transferase genotype; BMI, body mass index; FM, fat mass; FFM, fat free mass. Values are means ± standard deviations. Data were analyzed with a one-way ANOVA.

At baseline, EE **(Figure 5.2A)**, RQ **(Figure 5.2B)**, FATox and CHOox did not differ between genotypes, neither did they differ between treatments **(Table 5.4)**. Significant interactions were observed between COMT genotypes and treatment for RQ, FATox and CHOox ($p<0.05$) **(Table 5.5)**.

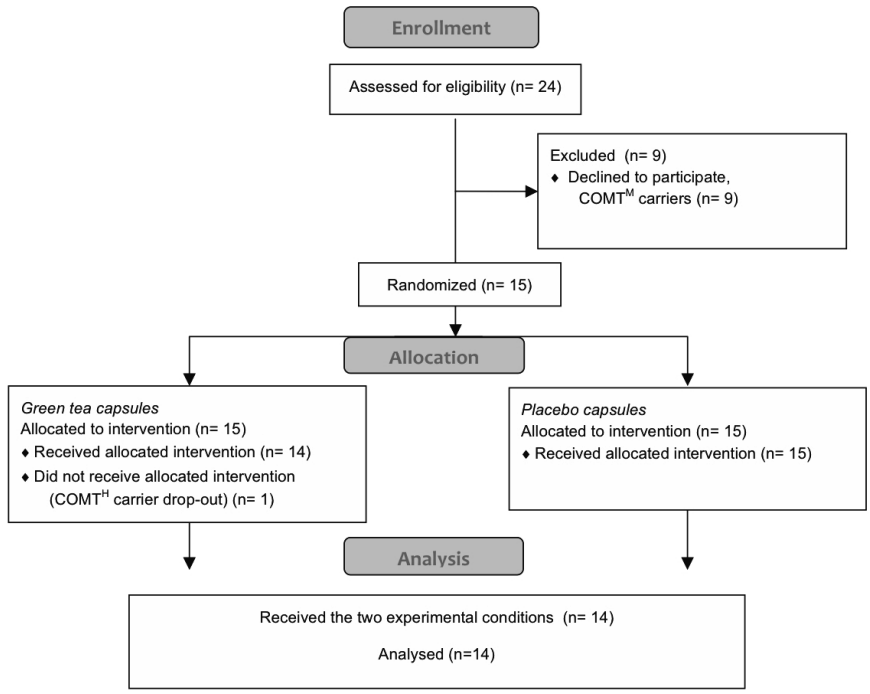

Figure 5.1 Flow diagram (CONSORT)

Table 5.3 Genotypic and allelic distribution.

Gene	SNP	G	F (N)	F (%)	Allele	F (%)	HWE
COMT	rs4680	GG *(Val/Val)*	8	33.3	G	52.1	0.22
		GA *(Val/Met)*	9	37.5	A	47.9	
		AA *(Met/Met)*	7	29.2			

SNP, single nucleotide polymorphism; G, genotype; F, frequency both absolute (N) and relative (%); COMT, catechol-O-methyl transferase. P-value obtained from the χ^2-test of Hardy Weinberg equilibrium.

Table 5.4 Baseline values of measured variables

	GT	PL	*P-values*	$COMT^H$	$COMT^L$	*P-values*
RQ	0.81 ± 0.03	0.81 ± 0.03	0.786	0.81 ± 0.03	0.81 ± 0.02	0.983
FATox (g/min)	17.1 ± 4.4	16.5 ± 4.9	0.558	16.9 ± 1.3	17.0 ± 1.2	0.994
CHOox (g/min)	20.4 ± 4.1	22.1 ± 6.3	0.684	21.4 ± 0.9	21.4 ± 2.0	0.963

EE, energy expenditure; RQ, respiratory quotient; FATox, fat oxidation; CHOox, carbohydrate oxidation; GT, green tea; PL, placebo; $COMT^H$, high activity catechol-O-methyl transferase genotype; $COMT^L$, low activity catechol-O-methyl transferase genotype. Values are means ± standard errors. Two-factor ANOVA with genotype ($COMT^H$ vs $COMT^L$) as factor 1 and treatment (GT vs. PL) as factor 2.

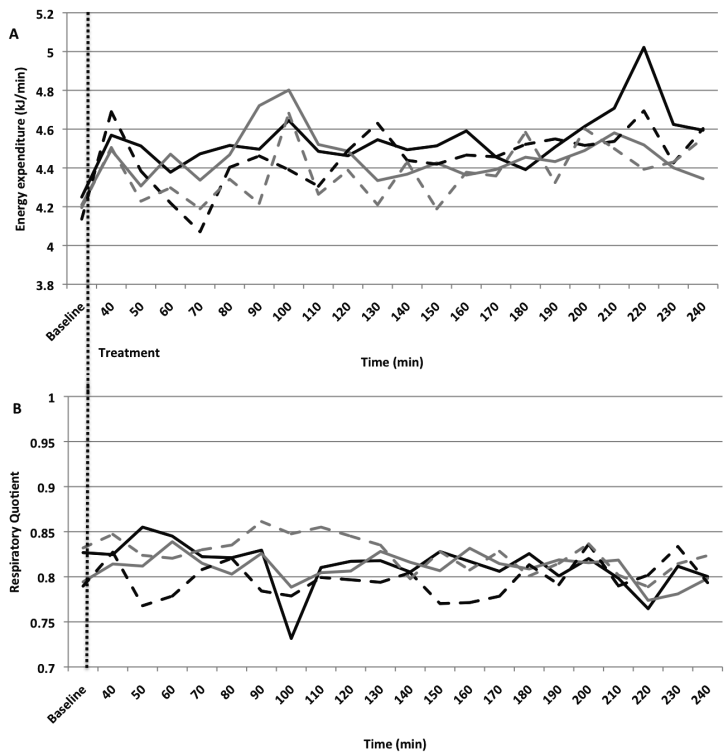

Figure 5.2 Real-time energy expenditure (EE; kJ/min, **A**) and RQ (**B**) before and after consumption of green tea (Black line) and placebo (Grey line) capsules in subjects carrying a $COMT^{L}$ genotype (Solid line) and $COMT^{H}$ genotype (Dotted line). Baseline resting energy expenditure and RQ were measured during the first 30 minutes. The vertical dotted line indicates the time that the treatment was given. Values are means (two-factor ANOVA with genotype as factor 1 and treatment as factor 2).

Also, when comparing the difference between GT and PL for genotypes, the delta was significantly larger in the $COMT^{H}$ genotype vs. $COMT^{L}$ genotype for EE (26.8±8.8 vs. 8.63±8.6 kJ.3.5hrs; $p<0.05$), RQ (-0.03±0.03 vs. 0.002±0.02; $p<0.001$), FATox (3.00±0.7 vs. 0.24±0.5 grams/d; $p<0.01$) and CHOox (-5.76±1.5 vs. 0.68±1.2 grams/d; $p<0.001$).
For treatment, significant differences were observed on EE, RQ, FATox and CHOox. In $COMT^{H}$ and $COMT^{L}$ groups combined, GT vs. PL increased EE (61.3±6.6 vs. 43.6±6.0 kJ.3.5hrs; $p<0.01$) and FATox, (17.8±0.5 vs. 16.2±0.4 g/d; $p<0.01$), while RQ (0.80±0.02 vs. 0.82±0.02; $p<0.01$) and CHOox (20.3±0.9 vs. 22.8±0.7 g/d; $p<0.03$) were decreased.

Table 5.5 Results for measured variables.

		EE (kJ/min)	RO	FATox (g/min)	CHOox (g/min)
ALL	GT	61.3 ± 6.6	0.80 ± 0.02	17.8 ± 0.5	20.3 ± 0.9
	PL	43.6 ± 6.0	0.82 ± 0.02	16.2 ± 0.4	22.8 ± 0.7
ALL	$COMT^H$	48.8 ± 5.8	0.81 ± 0.02	16.8 ± 0.4	21.4 ± 0.8
	$COMT^L$	56.0 ± 6.7	0.81 ± 0.01	17.2 ± 0.6	21.7 ± 1.0
$COMT^H$	GT	62.2 ± 8.0	0.80 ± 0.02	18.3 ± 0.5	18.5 ± 1.2
	PL	35.4 ± 7.7	0.83 ± 0.02	15.3 ± 0.5	24.3 ± 1.1
$COMT^L$	GT	60.3 ± 7.4	0.81 ± 0.03	17.3 ± 0.5	22.1 ± 1.4
	PL	51.7 ± 6.7	0.81 ± 0.02	17.0 ± 0.5	21.4 ± 0.9
		P-values	*P-values*	*P-values*	*P-values*
Treatment	GT PL	0.006	0.004	0.001	0.011
Genotype	$COMT^H$ $COMT^L$	0.259	0.958	0.452	0.727
Treatment*genotype		0.154	0.001	0.005	0.001

EE, energy expenditure; RQ, respiratory quotient; FATox, fat oxidation; CHOox, carbohydrate oxidation; GT, green tea; PL, placebo; $COMT^H$, high activity catechol-O-methyl transferase genotype; $COMT^L$, low activity catechol-O-methyl transferase genotype. Values are means ± standard errors. Two-factor ANOVA with genotype ($COMT^H$ vs $COMT^L$) as factor 1 and treatment (GT vs PL) as factor 2.

For subjects carrying the $COMT^H$ genotype, EE (GT: 62.2±8.0 vs. PL: 35.4±7.7 kJ.3.5hrs; p<0.01) (**Figure 5.3A**) and FATox (GT: 18.3±0.5 vs. PL: 15.3±0.5 grams/d; p<0.001) (**Figure 5.3C**) were significantly elevated after GT vs. PL, but not for subjects carrying the $COMT^L$ genotype (EE, GT: 60.3±7.4 vs. PL: 51.7±6.7 kJ.3.5hrs; NS), (FATox, GT: 17.3±0.5 vs. PL: 17.0±0.5 grams/d; NS). Correspondingly, RQ (GT: 0.80±0.02 vs. PL: 0.83±0.02; p<0.01) (**Figure 5.3B**) and CHOox (GT: 18.5±1.2 vs. PL: 24.3±1.1 grams/d; p<0.001) (**Figure 5.3D**) were significantly decreased after GT vs. PL for subjects carrying the $COMT^H$ genotype, but not for subjects carrying the $COMT^L$ genotype (RQ, GT: 0.81±0.03 vs. PL: 0.81±0.02; NS), (CHOox, GT: 22.1±1.4 vs. PL: 21.4±0.9 grams/d; NS).

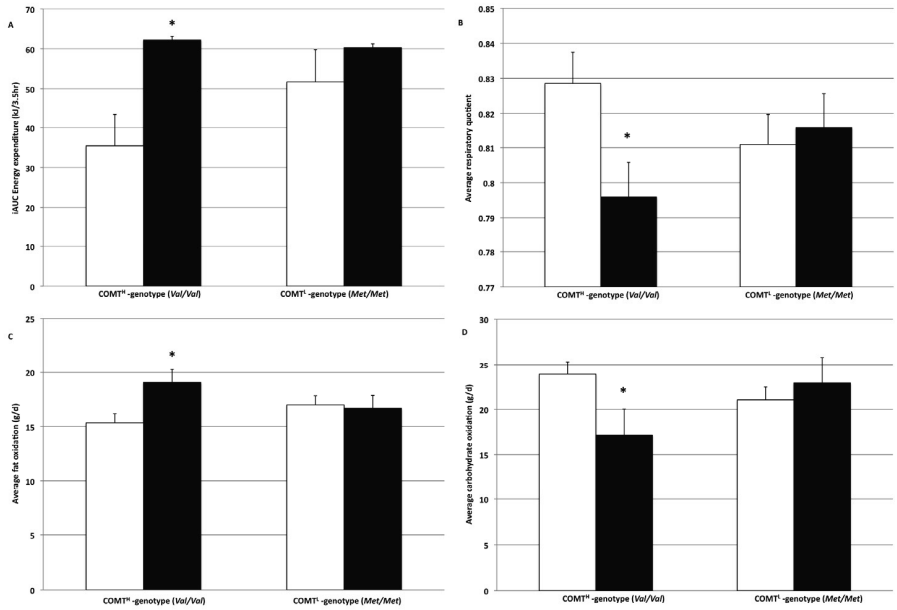

Figure 5.3 Results of the incremental area under the curve (iAUC) for energy expenditure (**A;** $p<0.01$), average respiratory quotient (**B**; $p<0.01$), average fat oxidation (**C**; $p<0.001$) and average carbohydrate oxidation (**D**; $p<0.001$) in the green tea (black) and placebo (white) conditions in fourteen subjects carrying either a $COMT^{H}$ genotype or $COMT^{L}$ genotype. Values are means, with standard errors represented by vertical bars. *Mean values for green tea were significantly different from placebo condition (two-factor ANOVA with genotype as factor 1 and treatment as factor 2).

Discussion

After investigating the effect of green tea and placebo consumption in subjects carrying different COMT genotypes, the results show that genetic predisposition may play an important role in whether or not subjects benefit from green tea. Significant interactions between treatment and COMT genotypes were observed for substrate oxidation. Also, the difference between GT and PL was much larger in the $COMT^{H}$ genotype carriers compared with the $COMT^{L}$ genotype carriers for EE, RQ, FATox and CHOox, which suggests a larger response of the $COMT^{H}$ genotype after GT ingestion. After treatment, it was observed that the impact of GT was more present in $COMT^{H}$ genotype carriers. These show a significant increase in EE and FATox as well as a significant decrease in RQ and CHOox that did not occur in the $COMT^{L}$ genotype carriers. Between genotypes, RQ and CHOox were significantly lower in the $COMT^{H}$

genotype carriers compared with the $COMT^{L}$ genotype carriers after GT vs. PL consumption. These findings may explain the variability as observed between subjects in response to GT administration, as well as the inconsistent results between studies investigating the beneficial effects of GT. It also shows that genetic predisposition may be a moderating factor as previously hypothesized in several manuscripts. The current results support the conclusions of a meta-analysis (10) that showed a seemingly smaller effect of catechins in Caucasian (-0.82 kg; 95% CI: -2.13 to 0.50) subjects compared with Asian subjects (-1.51 kg; 95% CI: -2.37 to -0.65). Jurgens *et al*. (11) drew a similar conclusion from their meta-analysis.

Remarkably, the interaction that we observed was partly due to subjects carrying the $COMT^{L}$ genotype having a higher PL induced EE and FATox, yet not an elevated GT induced EE compared with subjects carrying the $COMT^{H}$ genotype. Contrarily, the $COMT^{H}$ genotype carriers hardly increased EE and FATox after PL ingestion, while they did increase EE and FATox significantly after GT ingestion. Nevertheless, based on the previous, it may well be that studies including mostly subjects with the $COMT^{H}$ genotype, such as Asians, lead to more significant results by measuring a response which is not or less present in Caucasians that are more likely to be $COMT^{L}$ genotype carriers. This response may be modulated by SNS activity since COMT is a noradrenalin-degrading enzyme, with most likely different degrading rates between genotypes. However, methylation of catechins by COMT is not the only step in the conjugation process that precedes absorption. Glucuronidation and sulfation could potentially be susceptible to green tea catechins in a similar way as methylation. Phase II enzymes involved in these processes may lead to different outcomes as shown in studies examining the absorption and bioavailability of catechins. These are in line with intervention trials that report similar inconsistent results regarding the presence of metabolites in urine and plasma (18,19). Miller *et al*. (20) examined the effect of COMT genotype on absorption and metabolism of catechins and concluded that different polymorphisms seem to have no large impact. In contrast, Choi *et al*. (21) demonstrated in 660 daily GT drinking subjects that subjects with the low-activity COMT genotype excreted less catechin metabolites via their urine compared with subjects that carried the high-activity genotype. The absence of an effect in the study of Miller *et al*. (20) was attributed to the low availability of catechins. This was explained by the existence of two different COMT proteins; cytoplasm soluble protein (S-COMT) and membrane bound protein (MB-COMT). It appears that S-COMT has more affinity for metabolizing catechins, while MB-COMT metabolizes catecholamines. Nevertheless, it is debatable whether this makes any difference as S-COMT is the predominant form in most tissues and responsible for the majority of COMT enzyme activity, whereas only a small part of activity can be attributed to MB-COMT (22).

Moreover, Nackley *et al*. (23) suggested that, beside the *Val(108/158)Met* polymorphism, there are additional polymorphisms in the COMT gene that modulate

enzyme activity. Four polymorphisms in the COMT gene have been demonstrated to combine into 3 common haplotypes (24), which have been associated with variation in COMT enzyme activity (23). It should be taken into consideration that haplotype may account more for variability than an individual polymorphism and, therefore, play an important role in the effect of GT on EE and substrate oxidation.

Finally, it should be mentioned that the GT capsules contained a small amount of caffeine, which was not present in the PL capsules. Although it is not likely to have influenced the current results it should be taken into consideration. Usually, GT contains caffeine and the metabolism of caffeine may depend on the enzyme activity of *CYP1A1-CYP1A2* gene, which also differs between individuals (25).

Summarizing, subjects carrying the $COMT^{H}$ genotype may respond with increases of EE and FATox upon GT vs. PL ingestion, whereas $COMT^{L}$ genotype carriers react similarly to GT and PL ingestion. Green tea catechins appear to compensate for higher noradrenalin degradation in the $COMT^{H}$ genotype by inhibiting COMT. However, whether genetic predisposition is also a moderator in the long-term is still not evident, warranting a large-scale study in different ethnic populations. Also, interactions of different *CYP1A1-CYP1A2* and COMT polymorphisms as well as the role of haplotypes in the effect of GT on EE and substrate oxidation should be studied in the future.

In conclusion, subjects carrying the $COMT^{H}$ genotype increased energy expenditure and fat-oxidation upon ingestion of green tea catechins vs, placebo, whereas $COMT^{L}$ genotype carriers reacted similarly to GT and PL ingestion. The differences in responses were due to the different responses on PL ingesting, pointing to different mechanisms. The different alleles of the functional COMT *Val108/158Met* polymorphism appear to play a role in the inter-individual variability for EE and FATox after GT treatment.

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Chapter 6

Long-term green tea extract supplementation does not affect fat absorption, resting energy expenditure and body composition in adults

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Abstract

Introduction
Green tea extract (GT) may play a role in body weight regulation. Suggested mechanisms are decreased fat absorption and increased energy expenditure.

Objective
We examined whether green tea supplementation for 12 weeks has beneficial effects on weight control via a reduction in dietary lipid absorption, as well as an increase in resting energy expenditure.

Methods
Sixty Caucasian men and women (BMI: 18-25 kg/m^2 or >25 kg/m^2 age: 18-50 years) were included in a randomized, placebo-controlled design in which fecal energy content (FEC), fecal fat content (FFC), resting energy expenditure (REE), RQ respiratory quotient (RQ), body composition and physical activity were measured twice (baseline, vs. week 12). For 12 weeks, subjects consumed either GT (>0.56 g/d epigallocatechin-gallate + 0.28 ~ 0.45 g/d caffeine) or placebo (PL) capsules. Preceding the measurements, subjects recorded energy intake for four consecutive days and collected feces for three consecutive days.

Results
No significant differences between groups and no significant changes over time were observed for the measured variables. Overall means ± SDs were 7.2±3.8 g/d, 6.1±1.2 MJ/d, 67.3±14.3 kg and 29.8±8.6% for FFC, REE, body weight, and body fat percentage, respectively.

Conclusion
GT supplementation for 12 weeks in 60 men and women did not have a significant effect on FEC, FFC, REE, RQ and body composition.

Introduction

Green tea (GT) has been shown to have energy expenditure promoting effects, as it is rich in polyphenols, mainly catechins, which might play a role in body weight regulation. GT is made from the fresh leaves of Camellia sinensis L. and contains 10-20% catechins (1). The main GT catechins are epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG), with EGCG being the most abundant catechin. GT catechins have been reported to induce weight loss (2-6) and body fat loss (2,4,5) as well as to increase fat oxidation (7,8) and energy expenditure in short-term (7-10) and long-term studies (11). Inhibition of catechol-O-methyltransferase (COMT) by catechins has been proposed to be the principal mechanism behind these effects (12). GT also contains caffeine, which stimulates thermogenesis and fat oxidation (13,14) via inhibition of the enzyme phosphodiesterase (15). Thermogenic effects of GT have been shown to be the result of a combined effect from catechins and caffeine (7).

However, another important mechanism behind the anti-obesity effects of GT catechins, may be a decrease in dietary fat absorption, as inferred from increases in fecal fat excretion in rats (16-18). Also in humans, an increased fecal fat excretion after a 10-day treatment with a catechin-enriched beverage was observed (19). Catechins may attenuate lipid emulsification, which in the absence of catechins reduces lipid droplet size and increases the number of droplets thus improve the digestion of triglycerides by lipases. However, catechins may also inhibit the activity of gastric and pancreatic lipases (20). Therefore, impeding both, lipid emulsification and lipid digestion might cause a decrease in dietary fat absorption, which results in an increase in fecal fat excretion, thereby reducing digestion efficiency.
At present, most studies assessing the mechanisms of GT catechins related to treatment of obesity are related to sparing fat-free body mass and sustaining energy expenditure despite a negative energy balance (21,22). Here, we investigated possible prevention of overweight, assessing long-term effects of catechin- and caffeine-rich GT on dietary fat absorption in humans. We examined whether GT supplementation for 12 weeks has beneficial effects on weight control via a reduction in dietary lipid absorption, as well as an increase in resting energy expenditure (REE). Timing of the study was based upon several animal studies in which fat and energy absorption was measured over the long-term (16-18). To examine the dietary lipid absorption we assessed fecal fat content and fecal energy content. Physical activity was measured to assess whether subjects maintained their habitual activity. We hypothesized that a 12-week GT supplementation of nine GT capsules/d (>0.06 g EGCG + 0.03 ~ 0.05 g caffeine per capsule) in energy balance increases fecal fat excretion and REE resulting in a possible decrease in body weight.

Subjects and methods

Subjects were recruited by advertisements on notice boards at Maastricht University and in local newspapers. Sixty-five healthy, normal weight (Body Mass Index (BMI) 18-25 kg/m^2) or overweight/obese (BMI ≥25 kg/m^2) Caucasian subjects were included in the study. Subjects were 11 men and 54 women, aged between 18-50 yrs. During the screening, subjects completed questionnaires related to health, smoking behavior, use of medication, alcohol and caffeine consumption, physical activity and eating behavior. Both men and women were included in order to possibly apply the findings to the general population.

All subjects were non-smoking, healthy, weight stable (weight change < 3kg during the last 6 months), were not using a more than moderate amount of alcohol (<10 consumptions/ week) or caffeine (<100 mg/d), and were not using antibiotics during the last 6 months. The Three Factor Eating Questionnaire (TFEQ) was used to determine eating behaviour (23). The TFEQ measures the 3 factors involved in eating behavior as follows: cognitive restraint of eating (factor 1), disinhibition (factor 2), and hunger (factor 3). Based on the median for the TFEQ scores in the south of the Netherlands, non-restrained eaters (<10 times factor 1) were selected, these are persons who are not consciously occupied with food and who are not energy restricted. TFEQ was only measured at baseline, as it was used to exclude restrained eaters from participation. Subjects were not taking medications except for the use of oral contraceptives in women. Subject sample size was calculated with an α of 0.05, a β of 0.95, and mean ± SD of 19.3±12.9 g for polyphenol-enriched oolong tea and 9.4±7.3 g for control, using total fecal lipid excretion (g/3 days) data from a previous study to calculate the effect size (25). Sample size was calculated as 25 subjects per group. Taking a drop-out into account, the sample size was finalized as 60 subjects. A written informed consent was obtained from all the participants. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and the Medical Ethics Committee of the Academic Hospital in Maastricht approved the study.

Study protocol

The study was conducted in a randomized, placebo-controlled, single blind design with two randomly sequenced experimental groups (GT and PL). Subjects visited the university two times (baseline, and 12 weeks). Subjects were asked to travel by public transport or car, in order to avoid physical activity that would increase REE. They arrived in fasted state at 08:30 h. They emptied their bladder before anthropometric and REE measurements. After baseline measurements subjects received either GT or PL in capsule form, which they had to consume daily for a period of six weeks. Six

weeks after baseline, subjects received new capsules, which they had to consume daily for the last six weeks of the intervention.

Dosage

After baseline measurements, subjects received either capsules with green tea extract (containing >0.06 g EGCG and 0.03 ~ 0.05 g caffeine per capsule, Sunphenon 70H-T Taiyo kagaku Co Ltd., Mie, Japan) or PL capsules (**Supplemental Table S6.1**), which they consumed daily for 12 weeks. Subjects were randomly divided into the two groups. They had to consume nine capsules per day: three capsules between breakfast and lunch, three capsules between lunch and dinner, and three capsules in more than 2 hours after dinner. Subjects were instructed not to consume the capsules simultaneously with their meals in order to prevent confounding effects of the macronutrients. The GT extract Sunphenon 70H-T was used (Taiyo Kagaku Co. Ltd.). Dutch BioFarmaceutics B.V. encapsulated the GT and PL components. The capsules with GT are intended for use as a dietary supplement. When specifying the PL capsules the green tea extract was replaced with microcrystalline cellulose (0.27 g per capsule in the GT capsules vs. 0.38 g in the PL capsules). Microcrystalline cellulose is refined wood pulp, and often used in vitamin supplement. The capsules all had the same appearance. Compliance to capsule intake was checked by asking subjects to return all remaining capsules after 6 and 12 weeks. The bioactivity of the GT capsules was tested previously, in a randomized crossover experiment by Hursel *et al.* (24) in which comparable GT from Sunphenon was used. The bioactivity of the GT was shown as energy expenditure significantly increased 3.5h after ingestion of GT vs. PL. In addition, to test the bioactivity of the actual GT capsules we first have tested GT induced thermogenesis. Before ingestion of the GT capsules, REE was measured during 20 minutes. REE was 4.0±0.7 kJ/min. After ingestion of three GT capsules energy expenditure was measured for another 3.5 hrs. Energy expenditure increased 0.3±0.1 kJ/min after ingestion of GT vs. placebo capsules and resulted in an increase of 8.5±1.7% compared to REE ($p<0.001$).

Body composition

Body weight was measured at baseline and after 12 weeks using a digital balance and height by a wall-mounted stadiometer. BMI was calculated as body weight (kg) divided by height (m) squared. Fat mass was determined by Bod Pod (Life Measurement, Inc) measurements (air displacement plethysmography) (25). Fat mass index (FMI) was calculated by fat mass (kg) divided by height (m) squared. BMI, % body fat and FMI were used to define body composition. Waist and hip circumferences were determined in standing position by a tape measure. Waist circumference was measured at the smallest circumference between rib cage and iliac

crest, and hip circumference at the level of the spina iliaca anterior superior. Accordingly, waist-to-hip ratio (WHR) was calculated by dividing waist by hip circumference. Both waist circumference and WHR were used to define different patterns of body fat distribution.

Resting energy expenditure

REE and respiratory quotient (RQ) were measured by means of an open-circuit-ventilated hood system. After 30 min of resting, REE was measured in the morning in a fasted state while lying supine for 30 min. Gas analyses was performed by a paramagnetic oxygen analyzer 0-25% (type 1155; Servomex, Crowborough Sussex) and an infrared carbon dioxide analyzer 0-1% (type 1520; Servomex). Calculation of REE was based on the equation of Weir (26). RQ was calculated as carbon dioxide produced/oxygen consumed. Fat and carbohydrate oxidation per 20 minutes was measured with the formula of Carpenter in Brouwer (27). Once per week, the reliability of the ventilated-hood system was validated with a methanol test, with methanol burning during 20 min.

Fecal fat content

Fecal samples were collected for the analysis of fat excretion. All feces excreted were collected in pre-weighed plastic containers, during three days before each visit because reference values based on 3x24h feces are representative for the mean feces production and values based on 1x24h are affected by individual fluctuations. Before analysis, the fecal samples were homogenized. For each subject, samples from the same period were pooled. Total fecal fat was analyzed according to the following principle. Triglyceride, an ester derived from glycerol and three fatty acids, was hydrolyzed with a 33% potassium hydroxide solution in ethanol. After hydrolysis, the added potassium hydroxide was neutralized with the use of hydrochloric acid. The de-esterified fatty acids were isolated by extraction with toluene and subsequently, were directly determined in toluene by acid-base titration with tetrabutylammonium hydroxide and bromothymol blue as indicator (28,29).

Fecal energy content

Before analysis, the fecal samples were weighed and freeze-dried. Fecal energy was obtained using an adiabatic bomb calorimeter (IKA-Werke GmbH & Co. Kg. calorimeter system C200). Oxidation took place in a closed volume (the bomb) at a well-defined initial temperature. The heat produced during oxidation increased the temperature of the bomb and the energy released during oxidation was calculated from this temperature rise. Bombs were calibrated by using benzoic acid before use. Digestive efficiency, expressed as % usable energy intake, was calculated as the

difference between energy intake and fecal energy content expressed as percentage of the energy intake.

Energy intake and food choice

Subjects were asked not to change their food intake pattern during the period of twelve weeks. They were instructed to abstain from GT and to use less than 100 ml caffeine-containing beverages per day. Furthermore they were asked not to use dairy products within two hours after/before ingestion of the capsules, as consumption of milk-protein inhibits the effect of GT on diet-induced thermogenesis (DIT) (30). During the four consecutive days before baseline subjects were asked to maintain their food intake pattern and to record all the food and drinks they consumed, in order to be able to consume exactly the same during the four days before their final measurements at week 12. Subjects were stratified by low/normal fat intake (≤35 En%) vs. high fat intake (>35 En%) to examine the effects of green tea based on dietary fat intake.

Physical activity

Physical activity was measured in a subgroup of 23 subjects. Subjects in this subgroup were randomly selected and stratified by gender, age and BMI. The subjects were instructed to maintain their habitual activity level throughout the entire study period. Physical activity was determined with the use of a DirectLife triaxial accelerometer for movement registration (Tracmor$_D$, Philips New Wellness Solutions) during two occasions (one week before baseline and week 11). The Tracmor is a small device (7 × 2 × 0.8 cm; 30 g), which measures accelerations in the antero-posterior, medio-lateral, and vertical directions of the trunk (31). Subjects wore the accelerometer during waking hours in a belt at the lower back. Physical activity level (PAL) was measured with a model developed to predict PAL. The regression equation was given by: PAL = $1.354 + 256 \times 10^{-9} \times$ Counts/d (32).

Statistics

The Statistical Package for the Social Sciences 20.0 (SPSS) was used to test the long-term effects of GT on fecal fat content, fecal energy content, REE, body composition and physical activity. Both groups were stratified according to the number of men and women, BMI, age and the differences between these groups were checked with the use of a factorial analysis of variance (ANOVA). P-values for these differences were, respectively, >0.90 for sex distribution and BMI, >0.80 for age, >0.50 for body weight. Repeated-measures ANOVA was used to determine possible differences in measurements between the two groups (GT vs. PL) and between the different time-points (baseline and 12 weeks after the initiation of the GT or PL), and the interaction-

effect; Group * Time effect. Changes within groups were compared between groups. GT given at the same specific dose likely has varying effects on metabolism and absorption in each individual. Therefore, individual results have been clarified by showing the number of subjects in which GT had a desirable effect (responders) vs. subjects in which this desirable effect of GT has not been found (non-responders). All statistical tests were two-sided and differences were considered statistical significant if $p<0.05$. Values are expressed as means and standard deviations.

Results

Subject characteristics

65 healthy subjects started the experiments; five subjects dropped-out due to scheduling problems. 60 subjects (50 women, 10 men) completed both test days (**Figure 6.1**). Subjects were randomized and stratified on BMI, age and gender. Groups did not significantly differ at baseline (**Table 6.1)**. There were no significant differences in the response to GT between men and women.

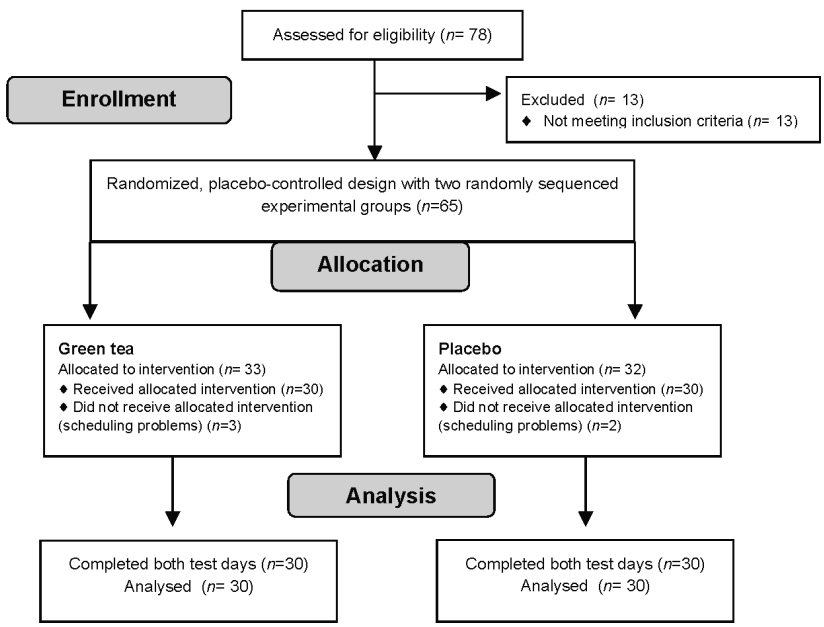

Figure 6.1 CONSORT flow diagram.

Table 6.1 Subject characteristics at baseline and week 12 in green tea and placebo groups (n=60)

	Green tea						Placebo					
	Men (*n*=5)		Women (*n*=25)		Total (*n*=30)		Men (*n*=5)		Women (*n*=25)		Total (*n*=30)	
	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12
Age, years	29.4±10.4	-	28.0±11.0	-	28.3±10.7	-	27.2±12.8	-	29.1±9.9	-	28.8±10.2	-
Height, m	1.80±0.06	-	1.67±0.06	-	1.69±0.08	-	1.81±0.07	-	1.66±0.07	-	1.69±0.09	-
TFEQ F1	4.4±2.3	-	5.8±3.0	-	5.5±2.9	-	4.6±3.4	-	5.3±2.5	-	5.2±2.6	-
TFEQ F2	2.6±1.8	-	4.2±1.7	-	4.0±1.8	-	3.0±1.6	-	3.6±2.2	-	3.5±2.1	-
TFEQ F3	1.2±1.1	-	3.9±2.7	-	3.4±2.7	-	3.2±2.5	-	3.4±2.4	-	3.4±2.4	-
Body weight, kg	72.9±10.1	71.7±9.3	66.0±15.4	66.1±15.7	67.1±14.7	67.1±14.9	86.1±18.9	87.1±19.2	63.5±9.1	63.4±9.5	67.2±13.8	67.4±14.4
BMI, kg/m^2	22.6±3.1	22.3±3.1	23.5±4.7	23.5±4.8	23.3±4.4	23.3±4.6	26.5±7.1	26.8±7.2	23.1±3.9	23.1±4.1	23.6±4.6	23.7±4.8
FMI, kg/m^2	4.6±2.5	4.5±2.4	7.8±3.4	7.8±3.6	7.3±3.4	7.3±3.6	7.3±4.9	7.2±4.8	7.4±3.2	7.5±3.3	7.4±3.5	7.5±3.5
FFMI, kg/m^2	18.0±0.7	17.8±0.7	15.7±1.7	15.7±1.6	16.1±1.8	16.0±1.7	19.3±2.4	19.6±2.8	15.7±1.2	15.5±1.2	16.3±2.0	16.2±2.2
WHR	0.85±0.10	0.85±0.10	0.75±0.11	0.75±0.10	0.77±0.11	0.77±0.10	0.85±0.11	0.86±0.08	0.70±0.05	0.70±0.05	0.73±0.08	0.73±0.08
FM, kg	14.7±7.8	14.3±7.5	21.9±10.1	22.1±10.7	20.7±10.0	20.8±10.6	23.6±14.5	23.2±14.0	20.1±7.7	20.5±8.1	20.7±8.9	21.0±9.1
FFM, kg	58.2±3.6	57.4±3.0	44.0±6.8	44.0±6.4	46.4±8.3	46.3±7.8	63.0±6.2	63.9±6.6	43.3±4.4	42.9±4.2	46.6±8.8	46.4±9.2
Body fat, %	19.5±7.2	19.2±7.2	32.0±7.3	32.0±7.9	29.9±8.6	29.9±9.1	25.5±10.7	25.0±10.3	30.9±7.9	31.4±8.1	30.0±8.5	30.3±8.7

[1] BMI: Body mass index; FMI: Fat mass index; FFMI: Fat free mass index; WHR: Waist-to-hip ratio; FM: Fat mass; FFM: Fat free mass. TFEQ: Three Factor Eating Questionnaire. The TFEQ measures three different factors of human eating behaviour. F1, cognitive restraint; F2, disinhibition; F3, hunger. [2] Values are means ± standard deviations. Data were analyzed by repeated-measures ANOVA. Groups did not differ significantly at baseline.

Compliance

Compliance was checked from counting the left-over and returned capsules. Subjects in the GT group returned 2.9±1.8 capsules/week and subjects in the PL group returned 3.0±1.7 capsules/week, without a difference in returned capsules between both groups.

Body composition

In subjects receiving GT body weight increased in 14/30 subjects, decreased in 15/30 subjects and in one subject body weight did not change between baseline and week 12. In the placebo-group body weight increased in 14/30 subjects, decreased in 14/30 subjects and did not change between baseline and week 12 in two subjects. No significant differences between groups (GT vs. PL) and no changes over time (baseline vs. week 12) were observed for body weight, body fat percentage and WHR (Table 6.1). There were no significant differences to the response to GT between subjects with BMI 18-25 kg/m^2 and subjects with BMI >25 kg/m^2 (**Supplemental Table S6.2).**

Resting energy expenditure

No significant differences were observed between GT (*n*=26) and PL (*n*=25) for REE, RQ, fat oxidation and carbohydrate oxidation (**Table 6.2**). With respect to possible responders and non-responders, in subjects receiving GT, 11/26 REE increased from week 0 to week 12, while in 15/26 REE decreased from week 0 to week 12. In the PL group 13/25 subjects REE increased from week 0 to week 12, while in 12/25 REE decreased over this period of time. Fat oxidation and carbohydrate oxidation (g/20 minutes) were not significantly different between GT and PL and no significant changes over time (baseline vs. week 12) were observed.

Fecal fat and energy content

Energy content of the dry stool did not differ significantly between GT and PL. No significant differences between groups (GT vs. PL) and no changes over time (baseline vs. week 12) were observed (**Table 6.2**). In the GT group fecal fat content increased in 18/30 subjects and decreased in 12/30 subjects and in the PL group fecal fat content increased in 16/30 subjects and decreased in 14/30 subjects. Fecal energy content and digestive efficiency (% usable energy intake) were not significantly different between GT and PL and no significant changes over time (baseline vs. week 12) were observed.

Table 6.2 Mean energy expenditure, fecal energy and fat content and energy and fat intake in the green tea and placebo groups at baseline and week 12.

	Green tea							Placebo						
	Men		Women		Total			Men		Women		Total		
	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12	*n*	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12	*n*
Resting energy expenditure, MJ/d	7.0±0.4	7.1±0.4	5.8±1.1	5.9±1.14	6.1±1.1	6.1±1.2	26	8.5±0.8	8.3±0.9	5.5±0.6	5.6±0.8	6.0±1.3	6.1±1.3	25
Respiratory quotient	0.86±0.04	0.83±0.04	0.83±0.05	0.84±0.05	0.84±0.05	0.84±0.1	26	0.86±0.06	0.83±0.05	0.84±0.06	0.81±0.05	0.85±0.06	0.82±0.05	25
Fat oxidation, g/20 min	0.7±0.3	1.1±0.3	0.8±0.3	0.8±0.4	0.8±0.1	0.8±0.1	26	1.4±0.9	1.2±0.4	0.8±0.4	0.8±0.4	0.8±0.1	0.9±0.1	25
Carbohydrate oxidation, g/20 min	2.8±0.6	2.2±0.9	1.6±1.0	1.9±1.2	1.9±0.2	2.0±0.2	26	2.6±2.8	2.7±1.4	1.6±0.8	1.5±0.8	1.7±0.2	1.7±0.2	25
Wet fecal weight, kg/d	0.14±0.07	0.14±0.07	0.11±0.05	0.10±0.05	0.11±0.05	0.11±0.06	30	0.12±0.04	0.11±0.09	0.12±0.05	0.11±0.05	0.12±0.05	0.11±0.05	30
Fecal fat content, g/d	5.9±2.4	7.2±3.0	6.6±3.1	7.5±4.8	6.5±3.0	7.5±4.5	30	6.9±5.5	9.4±3.6	7.8±3.6	6.7±3.7	7.7±3.9	7.2±3.8	30
Dry fecal energy content, kJ/g	21.9±1.5	21.5±0.6	22.8±1.9	22.8±1.9	22.6±1.8	22.6±1.8	20	22.5±1.6	22.6±1.2	22.5±1.4	22.9±1.7	23.3±1.5	22.8±1.6	21
Wet fecal energy content, kJ/g	5.9±2.7	5.8±2.0	6.7±2.2	6.1±1.4	6.5±2.3	6.0±1.5	20	6.3±1.3	5.1±0.9	7.4±1.9	6.3±1.5	7.1±1.8	6.0±1.4	21
Energy intake, MJ/d	10.6±2.2	10.6±2.2	7.5±2.7	7.5±2.7	7.9±2.0	7.9±2.0	20	9.3±2.4	9.3±2.4	7.7±2.5	7.7±2.5	8.2±1.8	8.2±1.8	21
Fat intake, En%	40.0±3.7	40.0±3.7	33.1±10.5	33.1±10.5	35.8±4.6	35.8±4.6	20	37.1±4.6	37.1±4.6	32.8±10.9	32.8±10.9	35.4±6.5	35.4±6.5	21
Digestive efficiency, %	91.4±5.0	90.4±7.2	91.0±4.7	89.7±5.3	91.0±4.7	89.7±5.3	20	92.3±3.9	90.5±7.1	86.6±10.4	89.0±4.8	88.0±9.5	89.3±5.2	21

[1] Values are means ± standard deviations. Data were analyzed by repeated-measures ANOVA. No significant differences between groups (GT vs. PL) and no changes over time (baseline vs. week 12) were observed. [2] Reported energy intake and fat intake were recorded during the 2 consecutive days before collection of the fecal energy content samples. [3] Digestive efficiency, expressed as % usable energy intake, was calculated as the difference between energy intake and fecal energy content expressed as percentage of the energy intake.

Energy intake and food choice

Mean reported energy intake and fat intake did not differ significantly between groups (**Table 6.3**). There were no significant differences to the response to GT between subjects with a relatively high fat intake (>35 En%) and subjects with a relatively low fat intake (≤35 En%).

Table 6.3 Mean reported energy and macronutrient intake per day in subjects with relatively low/normal (≤35 En%) and a high fat intake (>35 En%) in the green tea and placebo groups.

	Green tea (n=20)		Placebo (n=21)	
	≤35 En%	>35 En%	≤35 En%	>35 En%
n	10	10	10	11
Energy, MJ	7.2 ± 0.9	8.6 ± 2.6	7.5 ± 1.4	8.8 ± 2.0
Fat				
Fat, g	61.2 ± 9.8	91.5 ± 28.1	62.2 ± 17.2	93.5 ± 24.9
Fat, En%	31.9 ± 1.9	39.7 ± 2.6	30.5 ± 5.5	39.8 ± 3.4
Protein				
Protein, g	70.6 ± 13.8	73.7 ± 22.3	64.9 ± 17.8	76.1 ± 21.4
Protein, En%	16.4 ± 2.3	14.4 ± 1.5	14.5 ± 4.1	14.4 ± 1.2
Carbohydrate				
Carbohydrate, g	205 ± 24.4	225 ± 70.3	228 ± 50.8	221 ± 45.4
Carbohydrate, En%	47.8 ± 2.7	43.4 ± 3.6	50.7 ± 6.4	42.4 ± 3.0
Alcohol				
Alcohol, mg	2.8 ± 3.9	1.1 ± 3.5	3.5 ± 6.2	3.0 ± 5.4
Alcohol, En%	0.8 ± 1.1	0.3 ± 1.0	1.0 ± 1.8	0.9 ± 1.6
Dietary fibre, g	16.7 ± 4.3	18.1 ± 6.3	18.7 ± 5.6	18.7 ± 7.5
Minerals				
Sodium, g	2.7 ± 7.4	3.2 ± 0.9	2.6 ± 0.6	3.5 ± 1.0
Potassium, g	2.5 ± 0.5	2.6 ± 0.7	2.5 ± 0.6	2.7 ± 0.7
Calcium, g	0.7 ± 0.3	0.6 ± 0.2	0.6 ± 0.1	0.8 ± 0.3
Magnesium, g	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Iron, mg	8.6 ± 3.4	9.5 ± 3.6	9.2 ± 3.0	8.8 ± 3.6
Selenium, μg	38.0 ± 9.9	42.6 ± 17.5	33.9 ± 13.1	47.1 ± 17.6
Zinc, mg	8.2 ± 3.1	8.0 ± 1.7	7.9 ± 3.8	8.3 ± 3.8
Vitamins				
Vitamin A, mg	0.4 ± 1.4	0.8 ± 0.4	0.7 ± 1.0	0.8 ± 0.8
Vitamin B1, mg	1.0 ± 0.6	1.0 ± 0.3	0.8 ± 0.3	0.8 ± 0.3
Vitamin B2, mg	1.4 ± 0.8	1.1 ± 0.3	1.3 ± 0.7	1.1 ± 0.5
Vitamin B3, mg	15.7 ± 8.4	13.7 ± 3.6	14.1 ± 7.3	15.0 ± 6.4
Vitamin B6, mg	1.5 ± 0.8	1.3 ± 0.7	1.4 ± 0.7	1.3 ± 0.5
Folic acid, mg	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Vitamin B12, μg	3.9 ± 2.3	2.8 ± 0.9	3.2 ± 2.2	5.2 ± 5.8
Vitamin C, mg	78.7 ± 45.3	66.6 ± 25.7	71.8 ± 41.8	62.7 ± 28.1
Vitamin D, μg	2.2 ± 1.2	2.2 ± 0.8	1.8 ± 0.9	2.9 ± 2.0
Vitamin E, mg	10.5 ± 6.0	11.8 ± 3.9	8.3 ± 2.8	10.9 ± 4.4

[1] Energy intake and macronutrient intake were recorded during four consecutive days before baseline and week 12. [2] Data are means ± standard deviations. Data were analyzed by repeated-measures ANOVA. No significant differences between groups (GT vs. PL) were observed.

Physical activity

There were no significant differences between both groups at baseline. No significant differences between GT (week before baseline: 1.16±0.34, week 11: 1.10±0.30 kilocounts/min, *n*=13) and PL (week before baseline: 1.20±0.26, week 11: 1.04±0.28 kilocounts/min, *n*=10) and no changes over time (week before baseline vs. week 11) were observed. PAL was not significantly different between GT (week before baseline: 1.79±0.13, week 11: 1.75±0.12) and PL (week before baseline: 1.80±0.10, week 11: 1.74±0.10).

Discussion

Since no significant differences between groups (GT vs. PL) and no significant changes over time (baseline vs. week 12) were observed for body-weight, body-composition, resting energy expenditure, fat oxidation, and fat absorption, this study shows that long-term catechin- and caffeine-rich GT supplementation has no long-term effects in healthy normal weight and overweight subjects.

Nevertheless, previous studies, conducted in Asians, showed that administration of GT catechins for 12 weeks (2,5) or for 90 days (4) reduced body weight and body fat (2,4,5). However, in an 87-days study in Caucasians, no additional body-weight loss by administration of GT catechins was observed (33).
The difference between studies in Asian and Caucasian populations may be explained by differences in (I) body composition, as Asians have a higher body fat percentage compared to Caucasians with the same BMI (34), (II) in dietary habits, and (III) in genetic background. The latter may be explained by different SNPs of the functional COMT *Val108/158Met* polymorphism between ethnic groups, with a higher frequency of the high-activity enzyme, $COMT^{H}$-genotype (*Val/Val* polymorphism) in Asians, and a higher frequency of the low activity enzyme, $COMT^{L}$-genotype (*Met/Met* polymorphism) in Caucasians. This may play a role in the difference in sensitivity to green tea, with respect to energy expenditure and fat-oxidation (35). Yet, within the present GT-group the division between responders/non-responders is similar to that in the placebo-group, so this cannot be concluded from the present observation. The difference in variety in COMT enzyme activity between species, with higher activity in rats and lower activity in humans (36), could also explain the differences between the current study and the study of Raederstoff *et al.* (18).

With respect to GT-catechins induced energy expenditure, we confirmed the finding of previous studies showing an immediate increase over a short-term period (7-10), but we did not shown any increase in resting-energy expenditure over the long term.

Diepvens *et al.* showed that REE as a function of fat-free mass was maintained in the GT group, but significantly reduced in the PL-group, over 32 days (37). However, in this study subjects followed a low energy diet, which may imply that the effect of GT on REE is triggered by energy restriction. Only measuring REE in energy-balance, might be too weak to show an effect.
Similar to Diepvens et al. (37), we did not find an effect of GT catechin on fat oxidation in the long term. In short-term studies fat oxidation did increase (7,8), therefore the effect of green tea on fat oxidation seems to be acute. The dose in our study was higher than in the other studies (7,8,33), which might have caused habituation.
In another long-term study, Westerterp-Plantenga *et al.* (22) showed that a mixture of EGCG and caffeine was associated with greater weight maintenance in habitual low caffeine consumers, supported by relatively greater thermogenesis and fat oxidation. Here the difference with the present study is the design: weight maintenance after diet-induced weight loss, vs. possible weight loss only by GT administration.

In several animal studies beneficial effects of green tea polyphenols on fat absorption and body composition were found when combined with a high fat diet (18,38). With respect to the possible GT catechins induced reduction in fat absorption, Raederstorff *et al.* investigated the effect of GT catechins on lipid metabolism in rats when fed a high fat diet. They showed that EGCG increased fecal fat excretion (18). Likewise, Hsu *et al.* (19) found that polyphenol-enriched oolong tea increased fecal fat excretion when subjects consumed a high-fat diet. In the current study subjects were asked not to change their food intake pattern, therefore a possible explanation of the different results between the current study and the above-mentioned studies might be that the effect of catechins only occurs when combined with a high-fat diet. However, we also did not find significant differences to the response to GT between subjects with a relatively high fat intake (>35 En%) and subjects with a relatively low fat intake (≤35 En%). Furthermore, we used GT while Hsu et al. used oolong tea with polymerized-polyphenols. GT is non-fermented and non-oxidized, while oolong tea is semi-oxidized and semi-fermented. Most likely, there is a difference in catechin composition between the interventions in both studies. For instance, Hsu *et al.* (19) added polymerized polyphenols (0.21 g/d) to their tea beverages. The interplay between the different varieties of catechins may be of importance for the outcomes. Not only composition but also dosage differed. In the present study subjects received >1.35 g/d catechins (>0.56 g/d EGCG), which is relatively high compared to the dosage used in the study of Hsu *et al* (0.17 g/d catechins (0.03 g/d EGCG)) (19). Although it still remains controversial whether increasing dosage of EGCG leads to greater effect, the dosage might have contributed to the differences in results. The use of capsules with a green tea extract in this study versus tea beverages by Hsu et al. is another discrepancy, which may have had an effect on the bioavailability of catechins. Caffeine content of the beverages also differed between these studies; Hsu *et al* gave 0.13 g/d,

while caffeine content in our study was 0.28 ~ 0.45 g/d. The role of caffeine in fat absorption is not known yet. Caffeine does not have an effect on lipase activity (39). However, it has been shown that caffeine may increase colonic activity (40). This may shorten the presence of food in the gut, and shorter transit time decreased fat absorption (41-44).

Taken together green tea extract supplementation for 12 weeks did not have a significant effect on fecal energy content, fecal fat content, resting energy expenditure and respiratory quotient and may therefore not have lead to changes in body composition.

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Supplemental tables

Table S6.1 Composition of the green tea extract and placebo capsules and total dose consumed per day

	Green tea capsules		Placebo capsules	
	per capsule	per day	per capsule	per day
Capsules				
Total weight, g	0.65	5.82	0.51	4.60
Weight capsule, g	0.12	1.07	0.12	1.07
Weight filling, g	0.53	4.76	0.39	3.53
Filing				
Microcrystalline cellulose, g	0.27	2.43	0.38	3.44
Colloidal silicon dioxide. mg	5.50	49.5	7.80	70.2
Magnesium stearate, mg	2.60	23.4	2.60	23.4
Active filling, g	0.25	2.25		
Active filling				
Caffeine, g	0.03 ~ 0.05	0.28 ~ 0.45		
Polyphenols, g	0.20 ~ 0.22	1.80 ~ 1.97		
Polyphenols				
Catechins, g	>0.15	>1.35		
Other polyphenols, g	<0.07	<0.62		
Catechins				
Epigallocatechin gallate, g	>0.06	>0.56		

[1] Green tea extract: Sunphenon 70H-T (Taiyo Kagaku Co. Ltd., Mie, Japan). Dutch BioFarmaceutics B.V encapsulated green tea and placebo components. [2] Microcrystalline cellulose was used as filler, colloidal silicon dioxide and magnesium stearate were used as anti-caking agent. Capsules were made of hydroxypropyl methylcellulose. Titanium dioxide and copper complexes of chlorophyll were used as food coloring. [3] Subjects consumed nine capsules per day.

Table S6.2 Subject characteristics and measured variables in normal weight (BMI 18-25 kg/m^2) and overweight/obese (BMI >25 kg/m^2) subjects in the green tea and placebo groups at baseline and week 12.

	Green tea							Placebo						
	BMI 18-25 kg/m^2		BMI >25 kg/m^2		Total			BMI 18-25 kg/m^2		BMI >25 kg/m^2		Total		
	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12	*n*	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12	*n*
Age, years	26.3±9.8	-	33.6±12.1	-	28.3±10.7	-	30	25.9±8.4	-	36.9±10.7	-	28.8±10.2	-	30
Height, m	1.69±0.08	-	1.71±0.06	-	1.69±0.08	-	30	1.70±0.08	-	1.66±0.10	-	1.69±0.09	-	30
TFEQ F1	5.0±3.0	-	7.0±2.4	-	5.5±2.9	-	30	5.0±2.5	-	5.8±3.0	-	5.2±2.6	-	30
TFEQ F2	3.8±1.7	-	4.4±2.1	-	4.0±1.8	-	30	3.1±2.1	-	4.5±2.0	-	3.5±2.1	-	30
TFEQ F3	3.5±3.0	-	3.4±2.1	-	3.4±2.7	-	30	2.9±1.8	-	4.8±3.2	-	3.4±2.4	-	30
Body weight, kg	59.9±7.2	59.7±6.9	87.0±11.2	87.4±11.4	67.1±14.7	67.1±14.9	30	61.5±8.3	61.4±8.5	83.0±14.0	84.0±14.3	67.2±13.8	67.4±14.4	30
BMI, kg/m^2	21.0±1.3	20.9±1.4	29.7±3.5	29.9±3.7	23.3±4.4	23.3±4.6	30	21.3±1.7	21.2±1.7	30.2±3.7	30.5±3.9	23.6±4.6	23.7±4.8	30
FMI, kg/m^2	5.6±1.6	5.5±1.7	11.8±3.0	12.1±3.1	7.3±3.4	7.3±3.6	30	5.6±1.6	5.5±1.7	12.4±2.1	12.6±1.9	7.4±3.5	7.5±3.5	30
FFMI, kg/m^2	15.4±1.5	15.4±1.4	17.9±1.5	17.8±1.3	16.1±1.8	16.0±1.7	30	15.4±1.5	15.4±1.4	17.8±2.6	17.9±2.9	16.3±2.0	16.2±2.2	30
WHR	0.74±0.07	0.74±0.06	0.86±0.17	0.84±0.15	0.77±0.11	0.77±0.10	30	0.70±0.05	0.70±0.06	0.81±0.10	0.80±0.10	0.73±0.08	0.73±0.08	30
FM, kg	15.7±4.1	15.6±4.5	34.5±8.2	35.2±8.8	20.7±10.0	20.8±10.6	30	15.9±3.5	16.0±3.7	33.9±4.7	34.5±4.1	20.7±8.9	21.0±9.1	30
FFM, kg	44.2±7.7	44.1±7.3	52.6±6.8	52.2±6.1	46.4±8.3	46.3±7.8	30	45.6±7.7	45.3±8.1	49.4±11.2	49.5±11.8	46.6±8.8	46.4±9.2	30
Body fat, %	26.5±6.7	26.2±7.0	39.4±5.9	39.9±5.9	29.9±8.6	29.9±9.1	30	26.0±5.3	26.3±5.7	41.1±4.8	41.5±4.6	30.0±8.5	30.3±8.7	30
Resting energy expenditure, MJ/d	5.7±0.9	5.8±0.9	7.1±1.0	7.1±1.2	6.1±1.1	6.1±1.2	26	5.7±1.0	5.7±0.9	6.9±1.7	7.0±1.8	6.0±1.3	6.1±1.3	25
Respiratory quotient	0.83±0.05	0.84±0.05	0.84±0.05	0.86±0.05	0.84±0.05	0.84±0.1	26	0.84±0.07	0.83±0.06	0.88±0.06	0.81±0.07	0.85±0.06	0.82±0.05	25
Wet fecal weight, kg/d	0.11±0.06	0.11±0.06	0.12±0.04	0.12±0.05	0.11±0.05	0.11±0.06	30	0.11±0.05	0.11±0.05	0.13±0.07	0.11±0.07	0.12±0.05	0.11±0.05	30
Fecal fat content, g/d	6.5±3.3	7.3±4.6	6.5±1.9	8.0±4.2	6.5±3.0	7.5±4.5	30	7.8±3.7	7.5±4.1	7.4±4.4	5.8±2.7	7.7±3.9	7.2±3.8	30
Dry fecal energy content, kJ/g	22.3±1.5	22.3±1.4	22.7±2.3	23.3±3.2	22.6±1.8	22.6±1.8	20	23.4±1.3	23.1±1.4	22.9±1.7	22.2±2.0	23.3±1.5	22.8±1.6	21
Wet fecal energy content, kJ/g	6.5±1.7	6.1±1.6	6.4±4.2	5.6±0.9	6.5±2.3	6.0±1.5	20	6.8±1.6	5.9±1.5	7.7±2.1	6.5±1.3	7.1±1.8	6.0±1.4	21
Energy intake, MJ/d	7.8±2.4	7.8±2.4	8.5±2.4	8.5±2.4	7.9±2.0	7.9±2.0	20	7.6±2.2	7.6±2.2	9.3±1.5	9.3±1.5	8.2±1.8	8.2±1.8	21
Fat intake, En%	34.4±5.6	34.4±5.6	37.2±6.1	37.2±6.1	35.8±4.6	35.8±4.6	20	34.6±7.2	34.6±7.2	36.8±8.1	36.8±8.1	35.4±6.5	35.4±6.5	21
Digestive efficiency, %	91.5±4.9	89.9±5.6	88.9±5.8	89.0±5.0	91.0±4.7	89.7±5.3	20	88.5±10.2	88.6±4.9	87.7±8.7	92.1±5.3	88.0±9.5	89.3±5.2	21

[1] BMI: Body mass index; FMI: Fat mass index; FFMI: Fat free mass index; WHR: Waist-to-hip ratio; FM: Fat mass; FFM: Fat free mass. TFEQ: Three Factor Eating Questionnaire. The TFEQ measures three different factors of human eating behaviour. F1, cognitive restraint; F2, disinhibition; F3, hunger. [2] Reported energy intake and fat intake were recorded during the 2 consecutive days before collection of the fecal energy content samples. [3] Digestive efficiency, expressed as % usable energy intake, was calculated as the difference between energy intake and fecal energy content expressed as percentage of the energy intake. [4] Values are means ± standard deviations. Data were analyzed by repeated-measures ANOVA. No significant differences between groups (GT vs. PL) and no changes over time (baseline vs. week 12) were observed.

Chapter 7

Long-term green tea supplementation does not change the human gut microbiota

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Submitted for publication

Abstract

Introduction
Green tea (GT) catechins may play a role in body weight regulation through interactions with the gut microbiota.

Objective
We examined whether GT supplementation for 12 weeks induces changes in composition of the human gut microbiota.

Methods
58 Caucasian men and women were included in a randomized, placebo-controlled design. For 12 weeks, subjects consumed either GT (>0.56 g/d epigallocatechin-gallate + 0.28 ~ 0.45 g/d caffeine) or placebo (PL) capsules. Fecal samples were collected twice (baseline, vs. week 12) for analyses of total bacterial profiles by means of IS-profiling (IS-pro), a 16S-23S interspacer (IS) region-based profiling method.

Results
No significant differences between treatment groups and no significant changes over time were observed for body composition. Analysis of the fecal samples in subjects receiving GT and PL showed similar bacterial diversity and community structures, indicating there were neither significant differences in composition of the gut microbiota between groups nor significant changes over time (baseline vs. week 12). Although, there were no significant differences between normal weight and overweight subjects in response to GT, we did observe a reduced bacterial diversity in obese as compared to normal weight subjects.

Conclusion
GT supplementation for 12 weeks did not have a significant effect on composition of the gut microbiota.

Introduction

Green tea (GT) catechins have been shown to have anti-obesity effects in humans. In particular a decrease in body weight (1-5) and body fat loss (1,3,4) has been reported in response to GT catechins supplemention/consumption. Potential underlying mechanisms include the increase in energy expenditure, thereby preserving fat free body mass, and the promotion of fat oxidation (6-10). In addition a reduced fat absorption has been suggested (11), although evidence for long-term effects is lacking (12). Moreover, a role of gut microbiota has been suggested, possibly in relation to the preservation of fat free body-mass and the increase in energy expenditure (13).

The human intestinal microbiota constitutes a complex ecosystem, in which the majority of bacterial species belong to four phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*. The bacterial composition is rather stable within adults, whereas there is a large inter-individual variation (14). Over the past few years animal studies as well as human studies have suggested a role of the indigenous microbiota in body weight regulation (15). Body fat of germ-free mice increased after gut microbiota transplantation from normal mice, without an increase in food consumption (16), suggesting that the gut microbiota is important in regulation of energy harvesting and fat storage. Moreover, transplantation of the gut mcrobiota from obese mice into lean germ-free mice led to greater fat deposition than transplants from lean donors (17). Likewise, when fecal microbiota from obese and lean people was transplanted into germ-free mice, body weight only increased in mice receiving microbiota from obese people (18). Thus, the microbiota in the intestines of obese individuals may be more efficient in extracting energy from the diet and in storing this energy as fat, resulting in an increase in body weight and body fat percentage. Besides that, gut microbiota may also affect body weight regulation via metabolic signalling of short chain fatty acids through G-protein coupled receptors, to regulate appetite hormones and modulate inflammation (19). Several studies have actually found a difference in composition of the gut microbiota between normal weight and obese people (15,20,21), and have shown that bacterial diversity is reduced in obese compared with normal weight people (22,23). Although the composition of the human gut microbiota is considered to be essentially stable in adults, changes in diet may influence bacterial composition (24). Polyphenols for example, have a weight lowering effect (4,5,25), which is thought to be related to a change in the gut microbiota (13). Moreover, a major part of polyphenols may interact with the gut microbiota when they reach the intestines, as the bacteria in the intestines metabolize polyphenols (26,27). Furthermore, the cleavage of glycosidic linkages in polyphenols generates glycans that are important as a nutrient foundation for the gut microbiota (28) especially for the *Bacteroidetes*, as they are supposed to have a higher glycan degrading capacity than *Firmicutes*. Therefore polyphenols may

alter the balance between these two phyla in favour of *Bacteroidetes* (13). Furthermore, polyphenols may have a prebiotic effect as supplementation with polyphenols in obese subjects, with higher *Firmicutes/Bacteroidetes* ratio, was proposed for weight loss (29). The prebiotic effects of GT catechins on human gut microbiota are still poorly understood; only a few studies investigated the effect of catechins on the gut microbiota. Most of these studies found that catechin inhibits growth of certain pathogenic bacteria, including *Clostridium difficile* (30) and *Staphylococcus spp.* (31,32), *w*hile two studies found that catechins stimulate growth of beneficial commensal bacteria *Bifidobacterium spp.* (31,33,34). However, the long-term effect of catechins on energy extraction via a change in gut bacterial community structure is still unknown. Therefore, we investigated whether green tea induced changes in composition and diversity of the human gut microbiota. Furthermore we investigated whether there was a difference in composition of the gut microbiota between normal weight and overweight subjects at these levels and their possible differences in response to green tea.

Methods

Subjects

Subjects were recruited by advertisements on notice boards at Maastricht University and in local newspapers. Sixty-five healthy, normal weight (Body Mass Index (BMI) 18-25 kg/m^2) or overweight/obese (BMI ≥25 kg/m^2) Caucasian subjects, aged between 18-50 yrs were included in the study. All subjects were non-smoking, healthy, weight stable (weight change <3 kg during the past 6 months), not consuming a more than moderate amount of alcohol (<10 consumptions/week) and caffeine (<100 mg/day). Subjects were not using antibiotics during the last 6 months and were free of medication except for use of oral contraceptives in women. Furthermore subjects with Crohn's disease, ulcerative colitis or diverticulitis were excluded. Pregnant and lactating women were also excluded from participation.

Subject sample size was calculated using total fecal fat excretion data from a previous study to calculate the effect size (11), as this was also a primary endpoint of the study. Fecal fat excretion data were published elsewhere (12). Sample size was calculated as 25 subjects per group. Taking a drop-out into account, the sample size was finalized as 60 subjects. A written informed consent was obtained from all the participants. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and the Medical Ethics Committee of the Academic Hospital in Maastricht approved the study. The study was registered at clinicaltrials.gov as NCT01556321.

Study protocol

The study was conducted in a randomized, single blind, placebo-controlled design with two randomly sequenced experimental groups: GT or placebo (PL). Subjects visited the university twice (at baseline, and after 12 weeks). They arrived at fasted state at 08:30h and voided their bladder before anthropometric measurements. After baseline measurements, subjects received either GT or PL capsules, which they had to consume daily for a period of six weeks. Six weeks after baseline, subjects received new capsules, which they had to consume daily for the last six weeks of the intervention. They were instructed to abstain from GT and to use less than 100 ml. Subjects were asked to maintain their food intake pattern. During the four consecutive days before baseline they were asked to record all the food and drinks they consumed, in order to be able to consume exactly the same during the four days before their final measurements at week 12. They were instructed again to have the same diet during the four consecutive days before their visit in week 12 according to the recorded days before the baseline measurement **(Supplemental Table S7.1).** Subjects had to maintain their habitual activity level.

Dosage

After baseline measurements, subjects received either capsules with GT extract (containing >0.06 g Epigallocatechin-3-gallate (EGCG) and 0.03 ~ 0.05 g caffeine per capsule) or PL capsules (**Table 7.1**), which they consumed daily for 12 weeks. They had to consume nine capsules per day: three capsules between breakfast and lunch, three capsules between lunch and dinner, and three capsules in more than 2 hours after dinner. Subjects were instructed not to consume the capsules simultaneously with their meals in order to prevent confounding effects of the macronutrients (35). GT extract (Sunphenon 70H-T) was manufactured by Taiyo kagaku Co Ltd., Mie, Japan. Dutch BioFarmaceutics B.V encapsulated GT and PL components. The capsules with GT are intended for use as a dietary supplement. When specifying the PL capsules the GT extract was replaced with microcrystalline cellulose (0.27 g per capsule in the GT capsules vs. 0.38 g in the PL capsules). Microcrystalline cellulose is refined wood pulp, and often used in vitamin supplement. The capsules all had the same appearance. Compliance of capsule intake was checked by asking subjects to return all remaining capsules after 6 and 12 weeks. Bioactivity of the GT capsules was tested previously, in a randomized crossover experiment by Hursel *et al*. (35) in which comparable GT from Taiyo (Sunphenon) was used.

Table 7.1 Composition of the green tea extract and placebo capsules and total dose consumed per day

	Green tea capsules		Placebo capsules	
	per capsule	per day	per capsule	per day
Capsules				
Total weight, g	0.65	5.82	0.51	4.60
Weight capsule, g	0.12	1.07	0.12	1.07
Weight filling, g	0.53	4.76	0.39	3.53
Filing				
Microcrystalline cellulose, g	0.27	2.43	0.38	3.44
Colloidal silicon dioxide. mg	5.50	49.5	7.80	70.2
Magnesium stearate, mg	2.60	23.4	2.60	23.4
Active filling, g	0.25	2.25		
Active filling				
Caffeine, g	0.03 ~ 0.05	0.28 ~ 0.45		
Polyphenols, g	0.20 ~ 0.22	1.80 ~ 1.97		
Polyphenols				
Catechins, g	>0.15	>1.35		
Other polyphenols, g	<0.07	<0.62		
Catechins				
Epigallocatechin gallate, g	>0.06	>0.56		

[1] Green tea extract: Sunphenon 70H-T (Taiyo Kagaku Co. Ltd., Mie, Japan). Dutch BioFarmaceutics B.V encapsulated green tea and placebo components. [2] Microcrystalline cellulose was used as filler, colloidal silicon dioxide and magnesium stearate were used as anti-caking agent. Capsules were made of hydroxypropyl methylcellulose. Titanium dioxide and copper complexes of chlorophyll were used as food coloring. [3] Subjects consumed nine capsules per day.

Body composition

Body weight was measured at baseline and after 12 weeks using a digital balance and height by a wall-mounted stadiometer. BMI was calculated as body weight (kg) divided by height (m) squared. Fat mass was determined by Bod Pod (Life measurement, Inc) measurements (air displacement plethysmography) (36). Fat mass index (FMI) was calculated by fat mass (kg) divided by height (m) squared. BMI, % body fat and FMI were used to define body composition. Waist and hip circumferences were determined in standing position by a tape measure. Waist circumference was measured at the smallest circumference between rib cage and iliac crest, and hip circumference at the level of the spina iliaca anterior superior. Accordingly, waist-to-hip ratio (WHR) was calculated by dividing waist by hip circumference. WHR was used to define body fat distribution.

Microbiota

One day before the baseline visit and the visit in week 12, fecal samples were collected in pre-weighed plastic containers for analyses of the gut microbiota. Fecal

samples were collected by subjects at home, delivered at the University within 24h, and subsequently stored at -80°C until further processing.

DNA isolation

DNA was extracted from fecal samples with the easyMAG extraction kit according to the manufacturer's instructions (Biomérieux, Marcy l'Etoile, France). 100-400 mg of feces was placed in an Eppendorf tube with 200 µl of nucliSENS lysis buffer, and vortexed. Tubes were incubated shaking for 5 minutes at room temperature. After centrifugation (13000 rpm; 2 min), 100 µl of the supernatant was transferred to an easyMAG isolation container containing 2 ml of nucliSENS lysis buffer. This suspension was incubated for 10 min at room temperature after which 70 µl of magnetic silica beads were added. The easyMAG automated DNA isolation machine was used following the "specific A" protocol, eluting DNA in 110 µl buffer and stored at 4°C until further analysis.

IS-profiling of the intestinal microbiota

For IS profiling, DNA samples were diluted 1:10. Amplification of IS-regions was performed with the IS-pro assay (IS-diagnostics, Amsterdam, the Netherlands) according to the protocol provided by the manufacturer. IS-pro is a validated technique that combines differentiation bacterial species by the length of the 16S–23S rDNA intergenic spacer (IS) region with taxonomic classification by phylum-specific fluorescently labelled PCR primers (37). The procedure consists of two multiplex PCRs: the first PCR contains two different fluorescently labeled primers. One amplifying the phyla *Firmicutes*, *Actinobacteria, Fusobacteria* and *Verrucomicrobia (FAFV*) and the other labeled primer for the phylum *Bacteroidetes*. A separate PCR with a third labeled primer is performed for the phylum *Proteobacteria*. Amplifications were carried out on a GeneAmp PCR system9700 (Applied Biosystems, Foster City, CA). After PCR, 5 µl of PCR product was mixed with 20 µl formamide and 0.2 µl custom size marker (IS-diagnostics). Fragment analysis of the DNA was performed on an ABI Prism 3500 Genetic Analyzer (Applied Biosystems). IS-profiling resulted in microbial profiles. Each profile consisted of a set of color-labelled peaks; with each peak related to a specific IS fragment (measured in nucleotides) and the color related to a specific phylum group (*FAFV, Bacteroidetes* or *Proteobacteria*).

Data analysis

For data-visualisation of the bacterial composition, the Spotfire software package (TIBCO, Palo Alto, CA, USA) was used. All intensities were log2 transformed; this log2 transformation compacts the range of variation in peak heights, reduces the dominance of high peaks and includes less abundant species in downstream analyses.

This conversion was used in all downstream analyses, e.g. calculating between-sample microbial diversity. Microbial diversity was calculated both per phylum and for the overall microbial composition (by pooling all phyla together).

Alpha diversity and microbial profiles

Alpha diversity describes the within-sample bacterial diversity. To quantify and compare the alpha diversity the Shannon index was used (38). The Shannon diversity index is a diversity index that accounts for species abundance as well as the evenness of the species present. Diversity analysis was performed with the R 2.15.2 software package. First, we examined the microbial diversity in baseline samples in association to BMI; subjects were divided in two BMI categories (normal weight BMI 18-25 kg/m^2, overweight ≥25 kg/m^2), to determine differences in alpha-diversity between normal weight and overweight subjects. Then, we compared microbial diversity in baseline and week 12 samples of GT and PL.

A heat map was created by generating a correlation matrix of the log2 transformed profile data and hierarchical clustering of samples by the unweighted pair group method with arithmetic mean (UPGMA).

Beta diversity

Beta diversity captures the dissimilarity in bacterial composition between samples. Between-sample diversity, was analyzed by comparing the community structures, using the Bray-Curtis distance (39). Between-sample diversity was visualized by principal coordinates analysis (PCoA).

Statistical analysis

Groups were stratified according to the number of men and women, BMI and age. The Statistical Package for the Social Sciences 20.0 (SPSS) was used to test the effects of GT on body composition and microbial diversity, and to test the differences in microbial composition between normal weight and overweight subjects. The Mann-Whitney test, the nonparametric equivalent of the independent t-test, was used to determine differences in alpha-diversity between normal weight and overweight subjects at baseline. Repeated measures analysis of variance (ANOVA) was used to determine possible differences in body composition and microbial alpha diversity between the two groups (GT vs. PL).
Dissimilarity in microbial community structures (Beta-diversity) as measured by the Bray-Curtis index was compared between GT and PL, and tested using Mann-Whitney tests. All statistical tests were two-sided and differences were considered statistical significant if $p<0.05$. Values are expressed as means and standard deviations unless otherwise stated.

Results

Subjects

In this study, 65 subjects started the experiments; four subjects dropped-out due to scheduling problems and samples of three subjects were not included due to incomplete IS-profiles at baseline (one subject) or week 12 (two subjects). The final sample size was 58 subjects (46 women, 12 men). Groups were not significantly different at baseline with respect to age, sex and anthropometry **(Table 7.2).** No significant differences between groups (GT vs. PL) and no changes over time (baseline vs. week 12) were observed for body composition. Mean reported energy and fat intakes did not significantly differ between groups. Results on effects of GT on body composition and data on reported intake were published elsewhere (12). Furthermore, there were no significant differences in the response to GT between men and women. Compliance was checked from counting the left-over and returned capsules. Subjects in the GT group returned 2.9±1.8 capsules/week and subjects in the PL group returned 3.0±1.7 capsules/week, without a significant difference in returned capsules between both groups.

Diversity analysis

There was no significant difference between normal weight (BMI 18-25 kg/m^2) and overweight (BMI ≥25 kg/m^2) subjects in their response to GT. However, when baseline samples were compared, the Shannon diversity index indicated that the (alpha) diversity for all phyla combined was significantly lower in overweight subjects (*Mdn*=3.52, *n*=17) compared with normal weight (*Mdn*=3.78*, n*=43) subjects (*U*=180, *r*=-0.39, p=0.002, **Figure 7.1**).

Analysis of the fecal samples in subjects receiving GT and PL showed that the alpha diversity for all phyla combined as well as the diversity of *Bacteroidetes*, *FAVF* and *Proteobacteria* was not significantly different between groups (GT vs. PL) and also did not significantly change over time (baseline vs. week 12) (**Table 7.3**). A heat map was generated from all IS-profiles stratified by phyla. Absence of clustering was displayed in this heatmap as there was no separation by group and/or week (**Figure 7.2**)**.** Bray-Curtis index was used to assess the (dis)similarity in the microbial community structure between samples, the average Bray-Curtis dissimilarity (all phyla combined) of paired baseline and week 12 samples was not significantly different between GT and PL groups (**Figure 7.3**). This indicates that the (potential) change in microbiota composition during the intervention period was not significantly larger in subjects receiving GT as compared to PL.

Table 7.2 Subject characteristics at baseline and week 12 in the green tea and placebo groups (n=58).

	Green tea						Placebo					
	Men (*n*=6)		Women (*n*=24)		Total (*n*=30)		Men (*n*=6)		Women (*n*=22)		Total (*n*=28)	
	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12
Age, years	28.9±10.4	-	28.2±10.8	-	28.2±10.8	-	29.1±10.9	-	28.7±10.2	-	28.1±10.5	-
Height, m	1.71±0.09	-	1.70±0.08	-	1.70±0.08	-	1.70±0.10	-	1.70±0.07	-	1.70±0.09	-
Body weight, kg	68.9±14.6	68.9±14.7	66.8±14.1	66.7±14.2	66.8±14.1	66.7±14.2	69.2±14.3	69.3±14.9	63.2±9.0	63.1±9.4	67.5±14.0	67.8±14.5
BMI, kg/m^2	23.6±4.2	23.6±4.3	23.0±4.0	23.0±4.0	23.0±4.0	23.0±4.0	24.0±4.8	24.1±5.0	22.9±3.9	22.9±4.1	23.6±4.6	23.7±4.8
FMI, kg/m^2	7.1±3.4	7.1±3.5	6.9±3.1	6.9±3.2	6.9±3.1	6.9±3.2	7.5±3.7	7.6±3.8	7.3±3.3	7.5±3.4	7.2±3.5	7.4±3.6
FFMI, kg/m^2	16.5±2.0	16.5±1.8	16.1±1.9	16.1±1.8	16.1±1.9	16.1±1.8	16.5±2.1	16.5±2.3	15.6±1.2	15.5±1.2	16.3±2.0	16.3±2.2
WHR	0.76±0.10	0.76±0.08	0.76±0.09	0.76±0.08	0.76±0.09	0.76±0.08	0.74±0.09	0.74±0.09	0.70±0.04	0.70±0.05	0.73±0.08	0.74±0.09
FM, kg	20.6±9.6	20.6±10.1	19.9±8.9	19.9±9.2	19.9±8.9	19.9±9.3	21.3±9.4	21.5±9.6	19.9±7.8	20.3±8.3	20.4±9.0	20.7±9.3
FFM, kg	48.4±9.4	48.3±9.0	46.9±9.1	46.8±8.7	46.9±9.1	46.8±8.7	48.0±9.6	47.8±10.0	43.3±4.6	42.8±4.4	47.2±9.1	47.1±9.5
Body fat, %	29.1±9.0	29.0±9.4	29.1±8.2	29.1±8.5	29.1±8.2	29.1±8.5	30.1±9.3	30.3±9.5	30.7±8.2	31.2±8.5	29.5±8.7	29.7±9.0

[1] BMI: Body mass index; FMI: Fat mass index; FFMI: Fat free mass index; WHR: Waist-to-hip ratio; FM: Fat mass; FFM: Fat free mass. [2] Values are means ± standard deviations. Data were analyzed by repeated-measures ANOVA. Groups did not differ significantly at baseline.

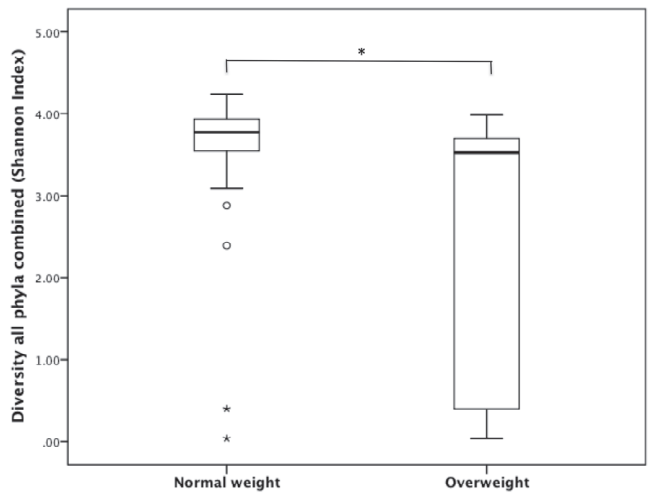


Figure 7.1 BMI categories: BMI 18-25 kg/m^2 (*Mdn*=3.78, *n*=43) vs. BMI ≥25 kg/m^2 (*Mdn*=3.52, *n*=17). All Phyla combined, Shannon diversity index using Mann-Whitney test, *p=0.002.

Table 7.3 Mean bacterial diversity per phylum in the green tea and placebo groups at baseline and week 12

	Group	Week	Diversity
All Phyla	GT	0	3.5 ± 0.2
		12	3.4 ± 0.2
	PL	0	3.1 ± 0.3
		12	3.4 ± 0.2
Bacteroidetes	GT	0	2.7 ± 0.2
		12	2.5 ± 0.2
	PL	0	2.6 ± 0.2
		12	2.6 ± 0.2
FAFV	GT	0	2.2 ± 0.2
		12	2.1 ± 0.2
	PL	0	2.2 ± 0.2
		12	2.0 ± 0.2
Proteobacteria	GT	0	2.1 ± 0.2
		12	1.9 ± 0.2
	PL	0	2.1 ± 0.2
		12	1.9 ± 0.2

[1] Green tea (GT, n=30) and Placebo (PL, n=28), FAFV: Firmicutes, Actinobacteria, Fusobacteria and Verrucomicrobia. [2] Shannon diversity index, Values are means ± standard errors. Data were analyzed by repeated-measures ANOVA. No significant differences between groups (GT vs. PL) and no changes over time (baseline vs. week 12) were observed.

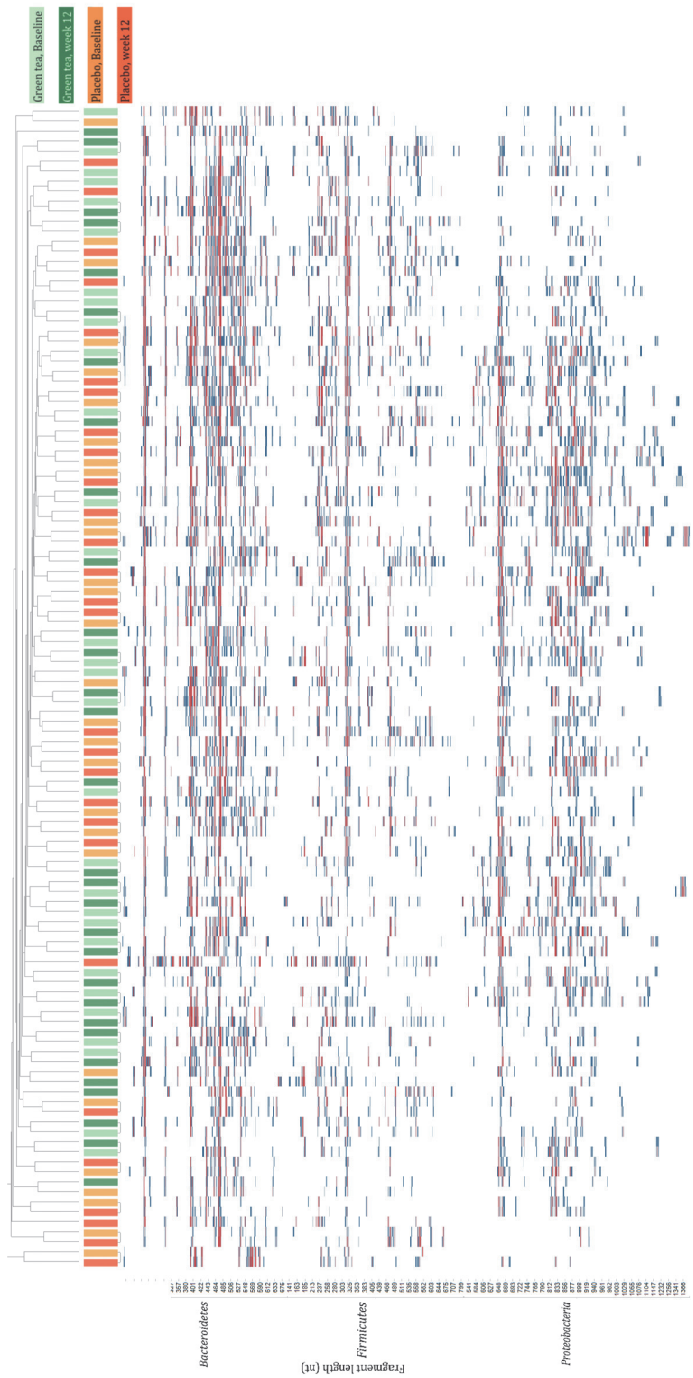


Figure 7.2 Heat map of all profiles sorted by phylum and clustered by the total profile, *n*=58. Green tea *n*=30, baseline (light green) and week 12 (dark green); placebo *n*=28, baseline (orange) and week 12 (red), 2LOG. Red signals represent dominant IS fragment lengths and blue signals represent less prevalent IS fragment lengths.

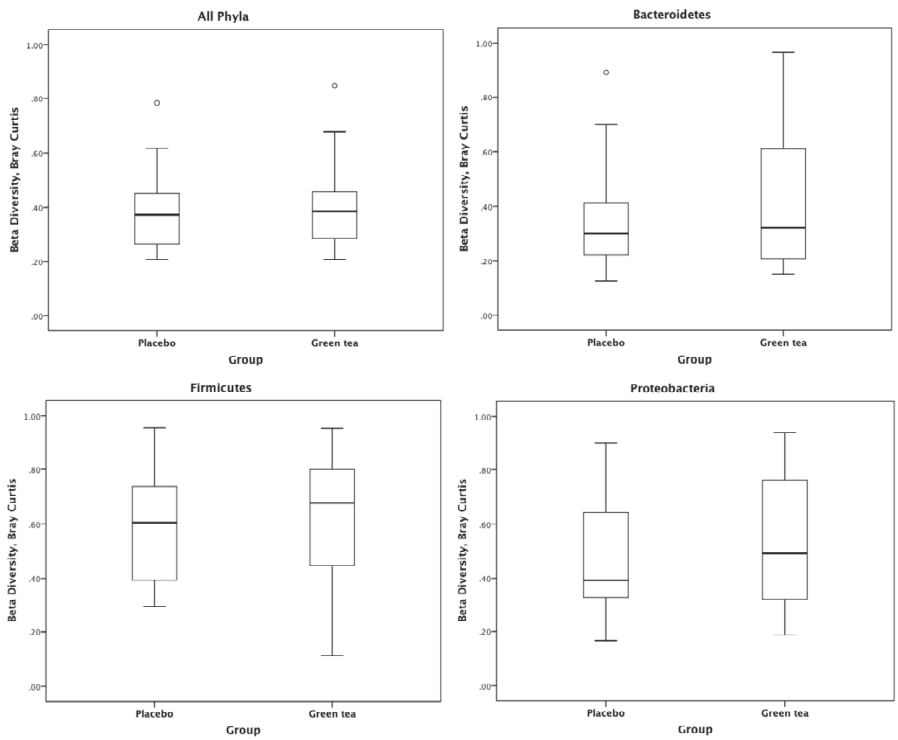
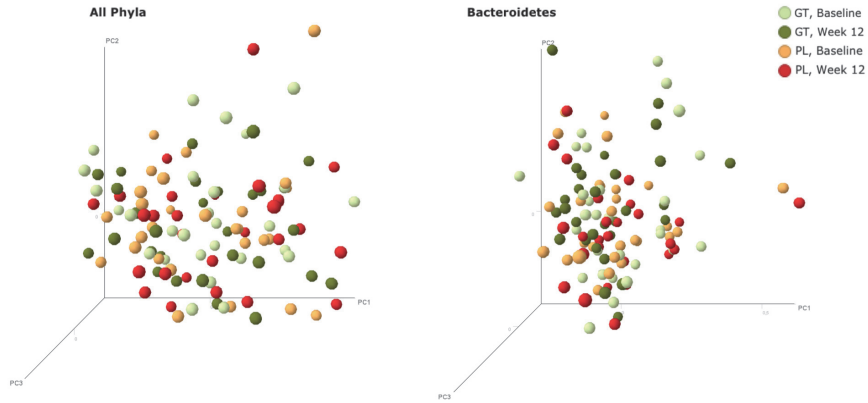


Figure 7.3 Box plots, comparisons of the between-sample diversity in green tea and placebo as calculated by Bray Curtis dissimilarity. Beta diversity captures the dissimilarity in microbial composition of the groups between baseline and week 12.

Moreover, clustering according to treatment group was neither observed when visualizing Bray Curtis distances using PCoA graphs for all phyla combined nor for the independent phyla *Bacteroidetes*, *FAFV* and *Proteobacteria* (**Figure 7.4**).



% Explained variance

Phylum	PC1	PC2	PC3	PC4	PC5
AllPhyla	0.09	0.05	0.05	0.04	0.03
Bacteroidetes	0.08	0.07	0.06	0.04	0.04
Firmicutes	0.10	0.08	0.06	0.04	0.04
Proteobacteria	0.11	0.08	0.05	0.04	0.04

Figure 7.4 Principal Coordinates Analysis (PCoA) plots of Bray Curtis distances between samples, with the %explained variance by the principle coordinates. PCoA per phylum, Bray Curtis, log2, n=58.

Discussion

In the present study no significant differences in the fecal bacterial diversity and community structure were observed between GT and PL, and also significant changes over time (baseline vs. week 12) were absent. Therefore, we conclude that catechin- and caffeine-rich GT supplementation has no long-term effects on composition of the gut microbiota in healthy normal weight and overweight subjects.

We did not observe a significant decrease in body weight and body fat percentage after 12 weeks of GT supplementation (12). However, another long-term study showed that a mixture of EGCG and caffeine was associated with greater weight maintenance, which was supported by relatively greater thermogenesis and fat oxidation (40). Therefore, it is likely that GT catechins only have beneficial effects on weight maintenance after weight loss (40), and these effects of catechins on body weight after a diet-induced weight loss may occur via a change in bacterial composition. This may as well be an explanation for the fact that we did not observe a difference in bacterial composition when subjects were in energy balance. In general, the gut microbial composition is quite stable in adults, therefore a change in body weight or extreme switches in dietary patterns may be needed to have a significant change in microbial composition (15,41). Furthermore, it is possible that the weight lowering effect of polyphenols only occurs in obese people (42). as supplementation with polyphenols in obese subjects with higher *Firmicutes/Bacteroidetes* ratio was proposed for weight loss (13).

Previous studies did find prebiotic effects of catechins on specific bacterial species. Most of these studies found that catechins may inhibit pathogenic bacteria and may stimulate beneficial bacteria (30-34). Apart from a study of Jin *et al,* in which an overall tendency for an increase in *Bifidobacteria* was found using qPCR (34), bacteria in the aforementioned studies were cultured, and as 60-80% of the bacterial species cannot be cultured most of the species were uncharacterized (43). Although, in these studies associations with several individual bacterial species were found, results on total bacterial composition and diversity were lacking. Furthermore, results of previous studies on catechins and gut microbiota are inconsistent in bacterial species that are associated with catechins. Our study is the first study to compare the human gut microbiota community structure and diversity after GT and PL supplementation by means of a comprehensive profiling method.

In the present study catechin- and caffeine-rich GT extract supplements were used. Although a favorable effect of catechin on gut microbiota was expected, the caffeine present may also affect the gut microbiota. However, only few studies investigated the effect of caffeine consumption on gut microbiota. A study in high-fat fed rats showed that caffeine attenuated the increase in *Firmicutes to Bacteroidetes* ratio, which normally occurs after a high-fat diet (44). In humans no effect of caffeine on the dominant bacteria in the intestines was found (45), these findings are consistent with the results of the current study.

There was no significant difference between normal weight and overweight subject in their response to GT. However, when subjects were divided in two BMI categories (normal weight and overweight), the overall microbial diversity was significantly lower

in obese as compared to normal weight subjects. These findings are in agreement with several previous studies that also indicated a reduced diversity in obese subjects (22,23). In a study with 154 subjects (including monozygotic and dizygotic twins) a reduced bacterial diversity was found in obese subjects compared with lean subjects (22).

This study has shown that long-term catechin- and caffeine-rich GT supplementation had no effect on composition of the gut microbiota and may therefore not have lead to changes in body composition, as no significant differences between conditions (GT vs. PL) and no significant changes over time (baseline vs. week 12) were observed for these measured variables. However we did find a reduced diversity in overweight and obese subjects compared with normal weight subjects. We conclude that green tea supplementation for 12 weeks does not have a significant effect on composition of the gut microbiota.

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Supplemental tables

Table S6.1 Composition of the green tea extract and placebo capsules and total dose consumed per day

	Green tea capsules		Placebo capsules	
	per capsule	per day	per capsule	per day
Capsules				
Total weight, g	0.65	5.82	0.51	4.60
Weight capsule, g	0.12	1.07	0.12	1.07
Weight filling, g	0.53	4.76	0.39	3.53
Filing				
Microcrystalline cellulose, g	0.27	2.43	0.38	3.44
Colloidal silicon dioxide. mg	5.50	49.5	7.80	70.2
Magnesium stearate, mg	2.60	23.4	2.60	23.4
Active filling, g	0.25	2.25		
Active filling				
Caffeine, g	0.03 ~ 0.05	0.28 ~ 0.45		
Polyphenols, g	0.20 ~ 0.22	1.80 ~ 1.97		
Polyphenols				
Catechins, g	>0.15	>1.35		
Other polyphenols, g	<0.07	<0.62		
Catechins				
Epigallocatechin gallate, g	>0.06	>0.56		

[1] Green tea extract: Sunphenon 70H-T (Taiyo Kagaku Co. Ltd., Mie, Japan). Dutch BioFarmaceutics B.V encapsulated green tea and placebo components. [2] Microcrystalline cellulose was used as filler, colloidal silicon dioxide and magnesium stearate were used as anti-caking agent. Capsules were made of hydroxypropyl methylcellulose. Titanium dioxide and copper complexes of chlorophyll were used as food coloring. [3] Subjects consumed nine capsules per day.

Table S6.2 Subject characteristics and measured variables in normal weight (BMI 18-25 kg/m^2) and overweight/obese (BMI >25 kg/m^2) subjects in the green tea and placebo groups at baseline and week 12.

	Green tea							Placebo						
	BMI 18-25 kg/m^2		BMI >25 kg/m^2		Total			BMI 18-25 kg/m^2		BMI >25 kg/m^2		Total		
	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12	*n*	Baseline	Week 12	Baseline	Week 12	Baseline	Week 12	*n*
Age, years	26.3±9.8	-	33.6±12.1	-	28.3±10.7	-	30	25.9±8.4	-	36.9±10.7	-	28.8±10.2	-	30
Height, m	1.69±0.08	-	1.71±0.06	-	1.69±0.08	-	30	1.70±0.08	-	1.66±0.10	-	1.69±0.09	-	30
TFEQ F1	5.0±3.0	-	7.0±2.4	-	5.5±2.9	-	30	5.0±2.5	-	5.8±3.0	-	5.2±2.6	-	30
TFEQ F2	3.8±1.7	-	4.4±2.1	-	4.0±1.8	-	30	3.1±2.1	-	4.5±2.0	-	3.5±2.1	-	30
TFEQ F3	3.5±3.0	-	3.4±2.1	-	3.4±2.7	-	30	2.9±1.8	-	4.8±3.2	-	3.4±2.4	-	30
Body weight, kg	59.9±7.2	59.7±6.9	87.0±11.2	87.4±11.4	67.1±14.7	67.1±14.9	30	61.5±8.3	61.4±8.5	83.0±14.0	84.0±14.3	67.2±13.8	67.4±14.4	30
BMI, kg/m^2	21.0±1.3	20.9±1.4	29.7±3.5	29.9±3.7	23.3±4.4	23.3±4.6	30	21.3±1.7	21.2±1.7	30.2±3.7	30.5±3.9	23.6±4.6	23.7±4.8	30
FMI, kg/m^2	5.6±1.6	5.5±1.7	11.8±3.0	12.1±3.1	7.3±3.4	7.3±3.6	30	5.6±1.6	5.5±1.7	12.4±2.1	12.6±1.9	7.4±3.5	7.5±3.5	30
FFMI, kg/m^2	15.4±1.5	15.4±1.4	17.9±1.5	17.8±1.3	16.1±1.8	16.0±1.7	30	15.4±1.5	15.4±1.4	17.8±2.6	17.9±2.9	16.3±2.0	16.2±2.2	30
WHR	0.74±0.07	0.74±0.06	0.86±0.17	0.84±0.15	0.77±0.11	0.77±0.10	30	0.70±0.05	0.70±0.06	0.81±0.10	0.80±0.10	0.73±0.08	0.73±0.08	30
FM, kg	15.7±4.1	15.6±4.5	34.5±8.2	35.2±8.8	20.7±10.0	20.8±10.6	30	15.9±3.5	16.0±3.7	33.9±4.7	34.5±4.1	20.7±8.9	21.0±9.1	30
FFM, kg	44.2±7.7	44.1±7.3	52.6±6.8	52.2±6.1	46.4±8.3	46.3±7.8	30	45.6±7.7	45.3±8.1	49.4±11.2	49.5±11.8	46.6±8.8	46.4±9.2	30
Body fat, %	26.5±6.7	26.2±7.0	39.4±5.9	39.9±5.9	29.9±8.6	29.9±9.1	30	26.0±5.3	26.3±5.7	41.1±4.8	41.5±4.6	30.0±8.5	30.3±8.7	30
Resting energy expenditure, MJ/d	5.7±0.9	5.8±0.9	7.1±1.0	7.1±1.2	6.1±1.1	6.1±1.2	26	5.7±1.0	5.7±0.9	6.9±1.7	7.0±1.8	6.0±1.3	6.1±1.3	25
Respiratory quotient	0.83±0.05	0.84±0.05	0.84±0.05	0.86±0.05	0.84±0.05	0.84±0.1	26	0.84±0.07	0.83±0.06	0.88±0.06	0.81±0.07	0.85±0.06	0.82±0.05	25
Wet fecal weight, kg/d	0.11±0.06	0.11±0.06	0.12±0.04	0.12±0.05	0.11±0.05	0.11±0.06	30	0.11±0.05	0.11±0.05	0.13±0.07	0.11±0.07	0.12±0.05	0.11±0.05	30
Fecal fat content, g/d	6.5±3.3	7.3±4.6	6.5±1.9	8.0±4.2	6.5±3.0	7.5±4.5	30	7.8±3.7	7.5±4.1	7.4±4.4	5.8±2.7	7.7±3.9	7.2±3.8	30
Dry fecal energy content, kJ/g	22.3±1.5	22.3±1.4	22.7±2.3	23.3±3.2	22.6±1.8	22.6±1.8	20	23.4±1.3	23.1±1.4	22.9±1.7	22.2±2.0	23.3±1.5	22.8±1.6	21
Wet fecal energy content, kJ/g	6.5±1.7	6.1±1.6	6.4±4.2	5.6±0.9	6.5±2.3	6.0±1.5	20	6.8±1.6	5.9±1.5	7.7±2.1	6.5±1.3	7.1±1.8	6.0±1.4	21
Energy intake, MJ/d	7.8±2.4	7.8±2.4	8.5±2.4	8.5±2.4	7.9±2.0	7.9±2.0	20	7.6±2.2	7.6±2.2	9.3±1.5	9.3±1.5	8.2±1.8	8.2±1.8	21
Fat intake, En%	34.4±5.6	34.4±5.6	37.2±6.1	37.2±6.1	35.8±4.6	35.8±4.6	20	34.6±7.2	34.6±7.2	36.8±8.1	36.8±8.1	35.4±6.5	35.4±6.5	21
Digestive efficiency, %	91.5±4.9	89.9±5.6	88.9±5.8	89.0±5.0	91.0±4.7	89.7±5.3	20	88.5±10.2	88.6±4.9	87.7±8.7	92.1±5.3	88.0±9.5	89.3±5.2	21

[1] BMI: Body mass index; FMI: Fat mass index; FFMI: Fat free mass index; WHR: Waist-to-hip ratio; FM: Fat mass; FFM: Fat free mass. TFEQ: Three Factor Eating Questionnaire. The TFEQ measures three different factors of human eating behaviour. F1, cognitive restraint; F2, disinhibition; F3, hunger. [2] Reported energy intake and fat intake were recorded during the 2 consecutive days before collection of the fecal energy content samples. [3] Digestive efficiency, expressed as % usable energy intake, was calculated as the difference between energy intake and fecal energy content expressed as percentage of the energy intake. [4] Values are means ± standard deviations. Data were analyzed by repeated-measures ANOVA. No significant differences between groups (GT vs. PL) and no changes over time (baseline vs. week 12) were observed.

Chapter 8

General discussion

In the studies described in this thesis, the potential beneficial effects of the thermogenic food ingredients capsaicin and green tea catechins on energy expenditure, energy intake, appetite, fat absorption and on interactions with gut microbial composition have been investigated.

Capsaicin

Energy expenditure and fat oxidation

In general, a negative energy balance achieved by energy restriction reduces energy expenditure (1) and increases appetite (2). For this reason we studied whether the effects of capsaicin in 25% negative energy balance counteracted these effects of the negative energy balance. Addition of 1.03 g of red chili pepper (2.56 mg capsaicin, 39,050 SHU) to the meals in 25% negative energy balance did prevent unfavorable effects of the negative energy balance on diet-induced energy expenditure and resting energy expenditure compared to a diet in energy balance without addition of capsaicin (3). Furthermore, when in the 25% negative energy balance condition capsaicin was added to the meals, fat oxidation was higher compared to the control condition in energy balance, while without addition of capsaicin fat oxidation was not significantly higher in 25% negative energy balance compared to the control condition. Additionally, in a 20% negative energy balance, capsaicin was also found to counteract the usual reduction in energy expenditure (4). In this study capsaicin and protein, singly as well as mixed, counteracted the unfavorable effects of the negative energy balance on energy expenditure. The meals that were offered had either a normal protein content of 10 En% or a high protein content of 25 En%; in the diets with a higher percentage of protein, carbohydrate was partly replaced by protein. The increase in energy expenditure was even larger when capsaicin was added to a diet with a higher percentage of energy from protein. In our studies energy percentage of fat was 30%, which was lower compared to a study of Yoshioka *et al* (5). In this study, the capsaicin induced increase in fat oxidation was suggested to be mainly due to the energy percentage of fat in the diet, being 45% (5). Most studies on effects of capsaicin on energy expenditure and fat oxidation were performed over the short-term (5-8). Only one study investigated the long-term effects of capsaicin on energy expenditure and fat oxidation. This study by Lejeune *et al* did not find a limiting effect of capsaicin on body weight regain after weight loss. Nevertheless, fat oxidation was relatively higher when capsaicin was added to the diet after four weeks of weight maintenance after weight loss (9). Moreover, resting energy expenditure was not decreased compared to baseline, while it was significantly lower in the placebo group after the four weeks of weight maintenance. This may be the result of adaptive thermogenesis, which is a decrease in resting energy expenditure beyond prediction

by loss of fat free mass and fat mass (10). As in subjects receiving capsaicin resting energy expenditure was not significantly different from baseline, this may suggest that capsaicin contributes to the prevention of adaptive thermogenesis. Taken together, capsaicin was able to counteract the unfavorable negative energy balance effect on energy expenditure, and to promote fat-oxidation beyond the normal increase, which occurs due to negative energy balance.

Energy intake and appetite profile

Another way in which capsaicin may affect body weight is through its effect on satiety and energy intake. Capsaicin was found to increase feelings of satiety and fullness in energy balance. In a negative energy balance (25% energy restriction) a decrease in desire to eat was observed after dinner (3), while with 20% energy restriction, capsaicin counteracted the decrease in feelings of fullness which was observed in the placebo condition with 20% energy restriction (4). When carbohydrate was partly replaced by protein (singly as well as mixed with capsaicin), feelings of fullness were even significantly larger in negative energy balance than in energy balance with normal protein content and without addition of capsaicin. In a previous study an acute beneficial effect of capsaicin on satiety and fullness in negative energy balance was found (11). In energy balance we also found that capsaicin tended to decrease *ad libitum* food intake (12). These findings agree with those of previous studies (13-16). A meta-analysis on effects of capsaicin on energy intake reported that consumption of at least 2 mg of capsaicinoids per day reduced energy intake by 74 kcal (310 kJ)/meal (17). In a study in which red pepper was ingested in capsule form as well as in tomato juice, the average daily energy intake was found to be 10% lower after the red pepper ingested in capsules and 16% lower after the red pepper in tomato juice compared to the placebo condition (13). This may reflect a decrease in appetite or a change in food choice, as subjects chose more food products with higher carbohydrate content and less food products with higher fat content during these capsaicin treatments. In summary, capsaicin increased feelings of satiety and fullness and tended to decrease *ad libitum* intake in energy balance. After dinner, capsaicin prevented the effects of the negative energy balance on desire to eat. The effects of capsaicin were even more pronounced in combination with a high-protein diet.

The subjects in our studies were used to consuming spicy foods on a regular basis. Yet, their consumption of spicy foods was still much lower than the consumption in Asian food patterns. In our studies the dosage of red chili pepper was 3.09 g (7.68 mg capsaicin) per day, which is relatively high for studies in Caucasians, while in studies with Asian subjects dosages are higher because red chili pepper is more common in Asian than in Caucasian food pattern (5,6,15). For instance the capsaicin consumption in India is 25–200 mg/day while the average daily consumption in Europe is estimated

to be 1.5 mg/day (18). This may affect results on appetite profile, energy intake, energy expenditure, and fat oxidation.

Green tea catechins

Genetic polymorphisms

Asians and Caucasians seem to respond differently to green tea catechins, as the effects on energy expenditure and body weight loss seem to be larger in Asian subjects (19,20). A meta-analysis of 11 clinical studies was undertaken to assess the effects of catechins on body weight loss and body weight maintenance (19). Although this meta-analysis showed that there is an effect of green tea catechins on body weight loss and weight maintenance after weight loss (standardized mean difference [SMD] -1.31 kg; 95% confidence interval [CI], -2.05 to -0.57; $p<0.001$), these effects seems to be smaller in Caucasians (SMD=−0.82 kg) compared with Asians (SMD=−1.51 kg). These ethnic differences can be explained by (I) differences in body composition, as Asians have a higher body fat percentage than Caucasians (21), (II) by dietary habits and (III) by genetic differences. Regarding the latter, the global variation in the COMT allele frequencies is of importance, as Asians have a higher frequency of the high activity $COMT^{H}$ allele and Caucasians have nearly equal frequencies of the $COMT^{H}$ allele and the low activity $COMT^{L}$ allele (22). Since COMT degrades catecholamines, these genotypes differ in COMT degrading capacity. In a study in which we compared the differences in effects of green tea catechins on energy expenditure and fat oxidation between $COMT^{H}$ allele carriers and $COMT^{L}$ allele carriers, we found that the effects of green tea catechins on energy expenditure, fat oxidation, carbohydrate oxidation and respiratory quotient were stronger in subjects carrying the $COMT^{H}$ allele (23). For these subjects, energy expenditure and fat oxidation were significantly higher after green tea supplementation compared with placebo, while we did not observe a significantly higher energy expenditure and fat oxidation in subjects carrying the $COMT^{L}$ allele. This may be an explanation for the beneficial effects of catechins on body weight, body composition and energy expenditure, that most of the studies conducted in Asians showed (24-30), while results of studies in Caucasians were inconsistent (31-34). Most likely these studies in Asians showed larger effects as presumably most of these subjects were carrying the $COMT^{H}$ allele, while in studies with Caucasian subjects there was a higher frequency of the $COMT^{L}$ allele.

Fat absorption

In addition to the effects of green tea catechins on energy expenditure and fat oxidation by inhibition of the enzyme COMT, catechins may as well have beneficial

effects on body weight control via a decrease in fat absorption. Previous animal and human studies suggest that green tea catechins might inhibit lipase activity, which is essential for fat absorption. Therefore, inhibition of lipase activity may cause a decrease in fat absorption (35,36), that consequently may result in an increase in fecal fat excretion (26,37). We investigated whether green tea with caffeine supplementation (>0.56 g/d epigallocatechin-gallate + 0.28-0.45 g/d caffeine) for 12 weeks has beneficial effects on body weight and body composition via a decrease in fat absorption in humans. We did not find a significant effect on body weight and body composition, nor on fecal fat excretion (38). Hsu *et al* did observe an increase of polyphenol-enriched oolong tea on fecal fat excretion when subjects had a 10-day treatment (26), and Raederstorff *et al* observed that green tea catechins increased fecal fat excretion in rats (36). However, the main difference between these studies and our study is the type of diet, as in our study subjects were asked not to change their food pattern while in the above-mentioned studies treatment was combined with a high-fat diet. Therefore the effect of green tea catechins may only become detectable when combined with a high-fat diet. A meta-analysis by Hursel *et al* showed a small but convincing effect of green tea catechins on body-weight loss and or body-weight maintenance (19). However, we did not find a significant effect of green tea catechins on body weight. Subjects in our study were asked not to change their food and activity pattern between baseline and visit in week 12. Consequently, possible effects of green tea catechins on body weight in energy balance may have been too weak to show an effect on body weight.

Energy expenditure

Furthermore, we did not find a significant effect of long-term green tea supplementation on resting energy expenditure and fat oxidation (38). This may be explained by the aforementioned difference in sensitivity to green tea catechins between Asians and Caucasians COMT enzyme, as only Caucasian subjects were included in our study. In our studies we did confirm the findings of previous studies on energy expenditure and fat oxidation over the short-term (39-42), as we observed an acute significant increase in energy expenditure 3.5 hours after green tea supplementation (23,38).

Gut microbiota: bacterial composition and diversity

Since several studies have shown that changes in the human diet can affect bacterial composition in the gut (43,44), and several studies suggested that the gut microbiota may play a role in the effects of polyphenols on energy extraction (45), we investigated whether green tea catechins induced changes in composition and diversity of the human gut microbiota. Analysis of the fecal bacterial diversity and

community structure of subjects receiving green tea catechins or placebo supplementation for 12 weeks did not show significant differences between both groups, nor between baseline and week 12 (46). Similar to the effects of green tea catechins on fat absorption, the effects of catechins on gut microbiota may mainly occur during weight maintenance after weight loss (33). Weight loss was previously been reported to increase the relative abundance of *Bacteroidetes* (47). It may be possible that first a weight-loss induced increase in diversity is necessary, which then may be sustained by effects of green tea catechins. As the composition of the gut microbiota is rather stable, a change in body weight or a more extreme switch in dietary pattern may be necessary to observe a significant effect on bacterial composition and diversity. Previous studies did find prebiotic effects of catechins on specific bacterial species, as catechins were found to inhibit pathogenic bacteria and to stimulate beneficial bacteria (48-52). However, in these studies associations with several individual bacterial species were found, while results on total bacterial composition and diversity were lacking. Furthermore, the significant prebiotic effects of catechins on specific bacterial species, which were found in these studies, may be spurious findings due to multiple testing. Furthermore, results of these studies with respect to the specific bacterial taxa associated with catechins were inconsistent. In our study fecal samples were analyzed using IS-profiling (IS-pro), which is a validated 16S-23S interspacer (IS) region-based profiling method to analyze the bacterial diversity and community structure (53). To summarize our findings, supplementation with green tea catechins for 12 weeks did not have a significant effect on composition and diversity of the gut microbiota.

Conclusions

Our most important finding on capsaicin consumption in relation to energy balance, is that in a negative energy balance, consumption of capsaicin helps to prevent weight cycling by sustaining energy expenditure and appetite profile, at a similar level as in energy balance. In energy balance, capsaicin increases fullness and satiety, and tends to prevent overeating, while in negative energy balance, capsaicin helps to prevent the yo-yo effect by sustaining diet-induced thermogenesis, resting energy expenditure and desire to eat after dinner similar to the level in energy balance. Furthermore it underscores improvement of body composition by showing a stronger increase in fat oxidation in negative energy balance than placebo treatment. The increase in fullness and energy expenditure was even larger when capsaicin was added to a diet with a higher percentage of energy from protein.

The main findings on the effects of green tea catechins were that the increase in energy expenditure and fat oxidation, decrease in carbohydrate oxidation and

respiratory quotient by green tea catechins were stronger in subjects carrying the $COMT^H$ allele than in subject carrying $COMT^L$ allele. Long-term supplementation with green tea catechins in energy balance did not have a significant effect on body weight, body composition, fat absorption, resting energy expenditure, fat oxidation and had no effects on composition of the gut microbiota.

Perspectives for future research

Since many people cannot consume capsaicin in large quantities because of its pungency, capsinoid the non-pungent analog of capsaicin, and nonivamide the less-pungent capsaicin analog may be more feasible to use. Capsinoid is found to increase energy expenditure and fat oxidation (54) and to reduce energy intake during positive energy balance (11). Nonivamide is also found to decrease energy intake, and is found to reduce feelings of hunger (55). It has been suggested that brown adipose tissue (BAT) has a role in the thermic effect of capsaicin and capsinoid, via stimulation of the TRPV1 receptor (56). Acute stimulation of TRPV1 by capsaicin elicits sympathetic nerve activation, which consequently leads to UCP1 dependent thermogenesis in BAT. BAT produces heat upon cold exposure and diet (57,58). A study on effects of capsinoids on energy expenditure and its relation to BAT activity showed that capsinoid increased the energy expenditure in subjects with metabolically active BAT (59), while this increase in energy expenditure was not observed in subjects in which metabolically active BAT was not shown. This suggests that the increase in energy expenditure in response to capsinoid (and possibly also capsaicin) may be dependent on presence of BAT and presumably activates BAT (56). Interestingly, Bakker *et al* (60) found differences in BAT volumes but not in total BAT activity between Asian and Caucasian people: the BAT volumes in people of South Asian decent were lower despite having equivalent BAT activity. Future research on effect of capsaicin on energy expenditure could focus on the presence of BAT and differences in effects of capsaicin or capsinoid between Caucasians and Asians.

Most studies on body weight maintenance after weight loss showed body weight regain (61-64). However, a meta-analysis by Hursel *et al* showed a small but convincing effect of green tea catechins on body weight maintenance (19). The effect of green tea extract on weight maintenance is dependent on the amount of caffeine subjects habitually consume, with a larger effect in habitually low caffeine consumers (<300 mg/d) than in habitually high caffeine consumers (>300 mg/d). In addition it is preferred to use a catechin and caffeine mixture, in which the caffeine is supposed to give an initial boost to the processing (33). Based on these studies it would be interesting to investigate whether a possible decrease in fat absorption and a change

in composition of the gut microbiota by consumption of green tea extract containing catechins and caffeine can prevent weight regain after weight loss. It has been shown already that weight loss is paralleled with a change in composition of the gut microbiota (47). Therefore, we speculate that green tea catechins may support this change in composition of the gut microbiota during weight maintenance after weight loss, and this possibly may be accompanied with a decrease in fat absorption. For the analyses of fecal fat excretion and bacterial diversity of the gut microbiota, we would collect fecal samples at baseline, after weight loss and after 12 weeks of weight maintenance. It would be interesting to test the hypothesis that green tea catechins may improve weight maintenance by preventing or limiting weight regain after weight loss via sustaining a weight-loss induced change in composition of the gut microbiota, paralleled by a decrease in fat absorption.

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Summary

Obesity is a result of an energy imbalance that develops when energy intake exceeds energy expenditure, and it is associated with increased morbidity and mortality. In an environment that is in abundance of easy accessible and energy-dense food, it is not easy to prevent a positive energy balance. Thermogenic ingredients, such as capsaicin in chili peppers and catechins in green tea, may promote energy expenditure and may decrease energy intake and absorption. Hence, we investigated whether these thermogenic food ingredients play a role in anti-obesity therapy.

Capsaicin

In general, weight loss decreases energy expenditure and increases appetite, which makes it more difficult to maintain body weight. This may consequently result in body weight regain. Previous studies suggested that capsaicin might decrease energy intake and promote energy expenditure in energy balance, while it adds only negligible amounts of energy itself. Therefore we investigated whether capsaicin can partly compensate for the impact of weight loss on the regulation of energy balance, and thus prevent the yo-yo effect or weight cycling. We investigated the effects of capsaicin on energy expenditure and substrate oxidation in healthy Caucasians in a respiration chamber in energy balance and in a 25% negative energy balance (75% of daily energy requirements (DER)). The main finding of this study was that in negative energy balance, when capsaicin was added to the meals, diet-induced thermogenesis and resting energy expenditure were similar to the level in energy balance, while these components of total energy expenditure were significantly lower in negative energy balance without addition of capsaicin to the meals. Furthermore in a 25% negative energy balance, capsaicin induced a larger fat oxidation compared with the control condition in energy balance, while fat oxidation was not significantly higher in negative energy balance without addition of capsaicin to the meals. With respect to the effects of capsaicin on appetite profile and energy intake, we found that capsaicin significantly increased feelings of satiety and fullness in energy balance. Furthermore, in energy balance there was a trend toward a decrease in energy intake when capsaicin was added to the diet. Without addition of capsaicin to the meals, desire to eat after dinner was significantly higher in negative energy balance compared with the control condition in energy balance, while capsaicin prevented these unfavorable effects of the negative energy balance on desire to eat. We also investigated the combined effects of capsaicin and a diet with a higher percentage of energy from protein. In this study we found that capsaicin consumption in a 20% negative energy balance (80% of DER) counteracted the effects on energy expenditure and feelings of fullness, and similar results were obtained with a diet with a higher percentage of energy from protein. However, the combination of capsaicin and a diet with a higher

percentage of energy from protein exceeded these effects. Taken together, capsaicin was able to counteract the unfavorable negative energy balance effects on energy expenditure and appetite profile. The increase in fullness and energy expenditure was even larger when capsaicin was added to a diet with a higher percentage of energy from protein.

Catechin

Previous studies reported larger effects of green tea catechins on energy expenditure and body weight in Asians compared with Caucasians. These differences can be explained by differences in body composition, dietary habits, and by genetic differences. We investigated these genetic differences in response to green tea catechins. Catechins inhibit the enzyme catechol-O-methyl transferase (COMT). COMT usually degrades catecholamines, therefore catechins may indirectly increase catecholamine secretion. Asians have a higher frequency of the $COMT^{H}$ allele, while Caucasians have nearly equal frequencies of the $COMT^{H}$ allele and $COMT^{L}$ allele. Since COMT degrades catecholamines, these genotypes may differ in capacity to degrade catecholamines. We showed different effects of green tea catechins on energy expenditure and fat oxidation between subjects carrying the high activity $COMT^{H}$ allele and subjects carrying the low activity $COMT^{L}$ allele. Subjects carrying the $COMT^{H}$ allele increased energy expenditure and fat oxidation upon green tea supplementation, whereas $COMT^{L}$ allele carriers reacted similarly to green tea and placebo capsules. This may explain why effects of green tea on energy expenditure and body weight loss seem to be larger in Asian subjects. In addition to these effects of green tea catechins on energy expenditure via inhibition of COMT, we were also interested in the effects of catechins on body weight control via a decrease in fat absorption, as previous studies suggested that green tea catechins inhibit the enzyme lipase, which is important in the absorption of fat. When fat absorption decreases, fecal fat excretion will increase. We investigated whether supplementation with green tea catechins for 12 weeks has beneficial effect on body weight and body composition via a decrease in fat absorption, and consequently an increase in fecal fat excretion. However we did not find a significant effect of green tea catechins on body weight, body composition and fecal fat excretion nor on resting energy expenditure and fat oxidation. Finally, as previous studies found prebiotic effects of green tea catechins, we were interested whether 12-week supplementation with green tea catechins may change the human gut microbiota. However, no significant differences in the fecal bacterial diversity and community structure were observed between baseline and week 12 in subjects receiving green tea. Our study has shown that long-term supplementation with green tea catechins has no effects on composition of the

human gut microbiota in normal weight and overweight subjects. Taken together, our findings on the effects of green tea catechins were that the different alleles of the COMT *Val108/158met* polymorphism play a role in the effects of green tea catechins on energy expenditure and fat oxidation. We also found that long-term supplementation with green tea catechins in energy balance did not have a significant effect on body weight, body composition, fat absorption, resting energy expenditure, fat oxidation and had no effects on composition of the gut microbiota.

Samenvatting

Obesitas ontstaat wanneer de energie-inname hoger is dan het energiegebruik. Obesitas wordt geassocieerd met een verhoogde morbiditeit en mortaliteit. In een omgeving waarin energierijke voeding in overvloed aanwezig is, is het niet makkelijk om deze positieve energiebalans te voorkomen. Thermogene ingrediënten, zoals capsaïcine, het pittige bestanddeel van chili pepers en catechine, de actieve component van groene thee, kunnen mogelijk het energiegebruik verhogen en de energie-inname of energie-opname in het lichaam verlagen. We hebben onderzocht hoe deze thermogene voedingsingrediënten een positieve energiebalans kunnen voorkomen en als zodanig zouden kunnen bijdragen aan het bevorderen van gewichtsafname bij obesitas, of het gewichtsbehoud na gewichtsafname.

Capsaïcine

Gewichtsvermindering heeft als nadelig gevolg dat het energiegebruik daalt en de eetlust stijgt. Dit maakt het lastig om op gewicht te blijven en niet weer opnieuw aan te komen. Eerdere studies hebben aangetoond dat capsaïcine in energiebalans het energiegebruik zou kunnen verhogen en de energie-inname zou kunnen verlagen. Vandaar dat we hebben onderzocht of capsaïcine gedeeltelijk kan compenseren voor deze nadelige gevolgen op de energiebalans bij gewichtsvermindering, en dus het jojo-effect kan helpen voorkomen. Bij de eerste studie hebben we de acute effecten van capsaïcine op het energiegebruik en de vetverbranding gemeten in een respiratiekamer, tijdens een negatieve energiebalans met 25% verminderde energie-inname (75% van dagelijkse energiebehoefte) en tijdens energiebalans (100% van de dagelijkse energiebehoefte). Uit dit onderzoek is gebleken dat in de controle conditie, dus zonder toevoeging van capsaïcine aan de voeding, de dieetgeïnduceerde thermogenese en het energiegebruik in rust significant lager zijn bij een verminderde energie-inname, en dat capsaïcine dit ongunstige effect van een verminderde energie-inname kan voorkomen. Verder heeft deze studie aangetoond dat bij een 25% verminderde energie-inname en toevoeging van capsaïcine aan de maaltijden, de vetverbranding significant hoger is dan bij de controle conditie in energiebalans zonder capsaïcine, terwijl deze bij een 25% verminderde energie-inname zonder capsaïcine niet significant hoger is dan de controle conditie. De resultaten met betrekking tot de effecten van capsaïcine op de eetlust in energiebalans waren zoals verwacht, aangezien we een significante verhoging van het verzadigingsgevoel hebben gevonden. Behalve deze effecten in energiebalans, liet deze studie zien dat de wens om te eten na de avondmaaltijd significant hoger is bij een verminderde energie-inname, en dat capsaïcine dit ongunstige effect van een verminderde energie-inname kan voorkomen. Naast de effecten van alleen capsaïcine, hebben we ook de effecten van capsaïcine in combinatie met een hoog-eiwit dieet onderzocht. Uit die

resultaten bleek, dat capsaïcine bij een 20% verminderde energie-inname (80% dagelijkse energiebehoefte) ervoor zorgt dat het energiegebruik en het verzadigingsgevoel niet omlaag gaan bij een verminderde energie-inname. Vergelijkbare resultaten werden gevonden bij het hoog-eiwit dieet. Echter, bij een hoog-eiwit dieet in combinatie met toevoeging van capsaïcine aan deze maaltijden, is het effect op het energiegebruik en op het verzadigingsgevoel nog groter. Concluderend hebben we met deze studies aangetoond dat capsaïcine in staat is om de nadelige effecten van een verminderde energie-inname op het energiegebruik en de eetlust tegen te gaan. De verhoging in het energiegebruik en het verzadigingsgevoel na toevoeging van capsaïcine aan de maaltijden is nog groter wanneer capsaïcine wordt toegevoegd aan een hoog-eiwit dieet.

Catechine

Eerdere studies hebben aangetoond dat effecten van catechine op het energiegebruik en op de lichaamssamenstelling groter zijn bij mensen van Aziatische afkomst, dan bij mensen van Kaukasische afkomst. Deze verschillen zouden kunnen worden verklaard door verschillen in lichaamssamenstelling, door andere voedingsgewoontes en door genetische verschillen. Het doel van onze studie was te kijken naar de genetische verschillen bij de effecten van groene thee op het energiegebruik en de vetverbranding. Catechine remt catechol-O-methyltranserase (COMT), dit is een enzym dat catecholamine afbreekt. Catechine zorgt dus indirect voor een toename in de catecholamine secretie. Onder de Aziatische bevolking is er een hogere frequentie van het hoge activiteit $COMT^{H}$ allel ten opzichte van de Kaukasische bevolking waar het $COMT^{H}$ allel en het lage activiteit $COMT^{L}$ allel even vaak voorkomen wat de discrepantie tussen Aziaten en Kaukasiërs kan verklaren omtrent de effecten van groene thee op het lichaamsgewicht en het energiegebruik. Aangezien COMT in staat is om catecholamine af te breken, is het mogelijk dat deze twee genotypes verschillen in het vermogen om catecholamine af te breken. Uit de resultaten van onze studie is gebleken, dat bij dragers van het $COMT^{H}$ allel het energiegebruik en de vetverbranding na groene thee consumptie stijgt, terwijl het energiegebruik en de vetverbranding bij dragers van het $COMT^{L}$ allel niet verandert door consumptie van groene thee. Dit sluit aan bij de eerder gevonden verschillen tussen Aziaten en Kaukasiërs, en zou het verschil in effecten van groene thee op het energiegebruik en gewichtsafname kunnen verklaren. Naast deze effecten van groene thee catechine op het energiegebruik via remming van COMT, hebben we onderzocht of catechine een gunstig effect heeft op het lichaamsgewicht door een verminderde vetopname in het lichaam. Eerdere studies hebben aangetoond, dat groene thee catechine het enzym lipase remt. Dit enzym is van belang bij de opname van vet in het lichaam. Wanneer

de vetopname vermindert, zal de hoeveelheid vet in de faeces toenemen. Vandaar dat we hebben onderzocht of groene thee op lange termijn gunstige effecten heeft op het lichaamsgewicht en de lichaamssamenstelling door een verminderde vetabsorptie, en door een toename van de hoeveelheid vet in de faeces. De data lieten echter zien dat groene thee geen effect heeft op het lichaamsgewicht, lichaamssamenstelling en op de hoeveelheid vet in faeces. Ook hebben we bij deze studie, terwijl het gebruikelijke effect van catechine op de dieet-geinduceerde thermogenese aanwezig was, geen effect op het energiegebruik in rust en de vetverbranding kunnen aantonen. Bovendien werd er in eerdere studies een mogelijk prebiotisch effect van groene thee catechine gevonden, vandaar dat we hebben onderzocht of catechine in 12 weken de microbiota in de darmen zou kunnen veranderen. We zagen geen verschillen in de bacteriële diversiteit in de darmen tussen baseline en na 12 weken groene thee supplementatie. We hebben dus niet kunnen aantonen dat groene thee catechine de microbiota in de darmen kan veranderen in 12 weken. Concluderend, bij dragers van het $COMT^{H}$ allel stijgt het energiegebruik en de vetverbranding na groene thee consumptie, terwijl dit bij dragers van het $COMT^{L}$ allel gelijk blijft, en groene thee heeft op lange termijn geen significante effecten op lichaamsgewicht, lichaamssamenstelling, vetopname, energiegebruik in rust, vetverbranding en heeft geen effect op de samenstelling van de microbiota in de darmen.

Valorisation

In the studies described in this thesis we investigated whether thermogenic ingredients, such as capsaicin in red pepper and catechins in green tea, have beneficial anti-obesity effects as they may induce body weight loss via an increase in energy expenditure, a decrease in appetite and fat absorption or a change in gut microbial composition. The valorisation potential of these studies in terms of economic and societal relevance, and implication for specific target groups will be described.

Treatment of obesity is beneficial, as weight loss reduces the risk of mortality and morbidity in obese subjects. Even modest weight loss leads to beneficial health effects (1-3). The reason for developing obesity is a positive energy balance i.e. a higher energy intake than energy expenditure. In an environment that is in abundance of easy accessible and energy-dense nutrition, it is not easy to change the energy balance (4-6). Investigating these thermogenic food ingredients, which may produce reductions in absorption and appetite, and promote energy expenditure and fat oxidation, has considerable importance for anti-obesity therapy. Therefore, investigating whether capsaicin and catechin may be helpful in reducing energy intake and energy absorption, and increasing energy expenditure and fat oxidation, is relevant to support body weight loss. Obese and overweight people who are willing to lose body weight can benefit from thermogenic food ingredients. Red pepper and green tea are widely used in many parts of the world. Green tea is appreciated for its characteristic and fresh taste and red peppers are used in meals for their pungency and aroma. Both red pepper and green tea are especially common in Asian countries.

Normally, losing weight by reducing energy intake causes a reduction in energy expenditure and an increase in appetite. We found that capsaicin can contribute to the prevention of the yo-yo effect when entering negative energy balance, by sustaining energy expenditure and appetite profile at a similar level as in energy balance. Thus, our studies suggest positive benefits for individuals in negative energy balance, as occurs when dieting. However, we expect that long-term intake of capsaicin during weight loss is required to produce a beneficial effect of capsaicin on body weight. Furthermore, we suggest that capsaicin can be used to increase satiety and fullness in energy balance. Chili peppers belong to the family Solanaceae, genus Capsicum, and the pungency is due to the presence of capsaicinoids, which are components of peppers. Capsaicin, dihydrocapsaicin, norcapsaicin, nordihydro-capsaicin, homocapsaicin and homodihydrocapsaicin are capsaicinoids, and capsaicin is the major pungent component of hot peppers. There are numerous different peppers of the genus Capsicum, which all differ in size, shape, colour and pungency. The pungency of peppers is measured in Scoville Heat Units (SHU), which varies from plant to plant. For example bell pepper is 0 SHU, as it is not pungent at all, while cayenne pepper is 30,000–50,000 SHU. Both peppers are commonly used in a variety

of cuisines. The red pepper used in our studies was 39,050 SHU, which is comparable with cayenne pepper. We suggest that a dose of 3 grams of red pepper per day (39,050 SHU) has beneficial effects on the energy balance. Dieticians can communicate this recommendation concerning usage of capsaicin in combination with a weight loss program. At present several companies produce products that contain capsaicin, such as encapsulated and powdered red pepper. Also the fresh, dried and powdered hot chili peppers available in supermarkets contain capsaicin, but with these products it is more difficult to determine the exact dosage consumed. Chili peppers can be ingested directly or in capsule form, with oral exposure being more effective. Therefore to achieve the maximum effect, oral exposure is needed (7). However, for long-term use capsules can be useful, as several doses per day are needed to achieve the maximum effect and because not everybody will enjoy to have a pungent meal several times per day. Although frequent users of capsaicin report the oral pungency as less pungent, there are still people that do not like their meals to be spicy and pungent. Another option is to use capsinoid, the non-pungent capsaicin analog, and novamide, the less-pungent capsaicin analog, as they are also found to have beneficial effects on energy intake, appetite and energy expenditure. Since we found that the effects of capsaicin on fullness and energy expenditure in negative energy balance are even larger in combination with a high protein diet, we suggest to keep the level of protein intake at 0.8-1.2 g/kg/d as it usually is in energy balance, and to reduce the carbohydrate and fat intake during energy restriction. This can be achieved by using food products high in protein such as meat, fish, milk, cheese, eggs, beans and nuts or by supplementation of the diet with a powdered form of protein, such as in protein shakes. To save time and effort it should be considered whether there is need for the food industry to develop new products, such as high-protein shakes with added capsaicin or capsinoids.

Results from animal and human studies show that green tea catechin might be a very promising food ingredient in lowering body weight, increasing energy expenditure and decreasing fat absorption. Several studies found beneficial effects of green tea catechins on body weight loss and body weight maintenance after weight loss. However, in our studies we did not find beneficial effects of green tea catechins on resting energy expenditure, fat absorption and gut microbiota over the long-term in energy balance. An explanation for the lack of significant effects of long-term green supplementation in energy balance may be due to the nature of the intervention. We did observe the usual short term increases in diet-induced energy expenditure. Yet for a sustained effect over the day and over the long term, green tea catechins are recommended to be used together with an energy restriction diet, in order to sustain energy expenditure at the original level. Moreover the effects on energy expenditure and body weight loss seem to be larger in Asian than in Caucasian subjects. Asians have a higher frequency of the $COMT^{H}$ allele and Caucasians have nearly equal

frequencies of the $COMT^H$ allele and $COMT^L$ allele. We found that the effects of green tea catechins on energy expenditure and fat oxidation were stronger in subjects carrying the $COMT^H$ genotype. So, most likely the studies in Asians show larger effects as presumably most of these subjects carry the $COMT^H$ allele. Therefore, there may be ethnic differences in the response to green tea catechins with respect to the anti-obesity effects. Green tea is made from the fresh leaves of Camellia sinensis L. and contains 10-20% catechins. The main green tea catechins are epicatechin (EC), epigallocatechin gallate (EGCG), epigallocatechin (EGC), and epicatechin gallate (ECG), with EGCG being the most abundant catechin. Green tea, white tea, oolong tea and black tea are all made from the leaves of the Camellia sinensis L and all contain catechins. However, green tea and white tea contain most catechins, as they are less processed than oolong tea and black tea. Soil quality, leaf age and growing condition affect the amount of EGCG in tea, therefore it is important to choose a high quality tea. In general, tea leaves and tea powders contain more EGCG than tea bags. Optimal time seems to be about 3 to 4 minutes in water that has boiled, but is reduced to a temperature of 85 degrees Celsius. Do not drink tea with milk, as milk takes away some health benefits nor with sugar because of its calorific value. With respect to the dosage, most studies used between 200 and 700 mg EGCG per day, and in our studies we used 560 mg EGCG mg per day. Nevertheless, the optimum dose and composition (caffeine and catechins) is unknown and it is uncertain whether increasing dosage leads to greater effect. At present several companies produce products that contain green tea catechins, such as encapsulated green tea extract. However, the availability of high quality green tea can be improved, as it is difficult to find high quality tea in places outside Asia.

Capsaicin and catechins are naturally occurring components found in plant products, which will be safe when individuals will not exceed the dosages used in our studies. To conclude, thermogenic food ingredients appear to have small effects, depending on the target groups.

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List of publications

Publications

Janssens PLHR, Penders J, Hursel R, Budding AE, Savelkoul PHM, Westerterp-Plantenga MS. Long-term green tea supplementation does not change the human gut microbiota. *Submitted for publication.*

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Janssens PLHR, Hursel R, Bouwman FG, Mariman ECM, Westerterp-Plantenga MS. The role of catechol-O-methyl transferase *Val108/158Met* polymorphism (RS4680) in the effect of green tea on fat oxidation and energy expenditure. Congress of the Society for the Studies of Ingestive Behavior 2012; Zürich, Switzerland. Poster presentation

Curriculum vitae

Pilou Janssens was born on April 25th 1986 in Heerlen, The Netherlands. After completing secondary school at Sophianum in Gulpen, she started the bachelor Nutrition and Dietetics at 'Hogeschool van Arnhem en Nijmegen', she obtained her bachelor's degree in 2008. From 2008 to 2011 she worked as a dietician in health care for people with intellectual disabilities at Stichting Pergamijn in Echt. In 2011 she obtained the degree of Master in Physical Activity and Health, with a specialization in Nutrition and Metabolism, for which she received an award for top 3% students at Maastricht University. In the same year Pilou started working as a research assistant at the Department of Human Biology at Maastricht University. In July 2012, she started her PhD research at the same department under supervision of Prof. dr. Margriet Westerterp-Plantenga. The research performed during this project is described in this thesis, entitled "Thermogenic ingredients; energy expenditure and intestinal absorption". During her PhD trajectory Pilou presented her results at national and international conferences.